# The Role of the Fas/FLIP Pathway in Insulin Secretion and Diabetes

DISSERTATION

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To my husband, Marc,
with all my love.
Thank you for your support.

# Summary

A decrease in functional insulin-producing pancreatic β-cells contributes to the establishment of not only type 1 but also of type 2 diabetes. Fas (CD95) is a cell surface receptor which plays a central role in the regulation of death in many cell types including β-cells and may be implicated in the development of type 1 and 2 diabetes. Glucose-induced β-cell apoptosis in human islets involves IL-1β mediated up-regulation of Fas and subsequent interaction with the constitutively expressed Fas ligand (FasL) on neighboring β-cells. Fas/FasL interaction leads to cleavage of procaspase-8, the most upstream caspase in the Fas apoptotic pathway. However, elevated glucose concentrations have a dual effect on β-cell turnover inducing proliferation in the short-term and apoptosis in the long-term . This dual effect of glucose may be explained by the pivotal role of FLICE-inhibitory protein (FLIP) on Fas signaling. Here we show that the Fas pathway also regulates β-cell secretory function. We observed impaired glucose tolerance in Fas deficient mice due to a delayed and decreased insulin secretory pattern. Expression of PDX-1, a β-cell specific transcription factor regulating insulin production and mitochondrial metabolism, was decreased in Fas deficient β-cells. As a consequence, insulin and ATP production were severely reduced and partly compensated for by a dramatic increase in β-cell mass. Up-regulation of FLIP enhanced NF-$\kappa$B activity via NF-$\kappa$B-inducing kinase and RelB. This led to increased PDX-1 and insulin production independent of changes in cell turnover. Several observations point to a role for the Fas pathway in the regulation of cell-cycle independent events including T cell activation, renal tubular epithelial integrin function, and protection against neurodegeneration. However, a direct interaction between the Fas pathway and PDX-1 has not been investigated.

Looking upstream of Fas, at IL-1β, we show that IL-1β at low concentrations may participate in the mitogenic actions of glucose through the Fas-FLIP pathway. Thus, exposure of human islets to low IL-1β concentrations (0.01–0.02 ng/ml) stimulated proliferation and decreased apoptosis whereas 2–5 ng/ml of IL-1β had the reverse effect. A similarly bimodal induction of FLIP, PDX-1 and Pax4 mRNA expression as well as glucose-stimulated insulin secretion was observed. In contrast, Fas induction by IL-1β was monophasic. Low IL-1β also induced the IL-1 receptor antagonist (IL-1Ra), suppression of which by RNA interference abrogated the beneficial effects of low IL-1β. The Fas antagonistic antibody ZB4 and siRNA to FLIP prevented low IL-1β-stimulated β-cell proliferation. Consistent with our in vitro results, IL-1β knockout mice displayed glucose intolerance, similar to that seen in the Fas-deficient mice, along with a decrease in islet Fas, FLIP, Pax4 and PDX-1 transcripts. These findings indicate that low IL-1β levels positively influence β-cell function and turnover

through the Fas-FLIP-pathway, and that IL-1Ra production prevents harmful effects of high IL-1β concentrations.

Type 2 diabetes is characterized by a deficit in β-cell mass and its incidence increases with age. In cultivated human islets, age correlated positively with the sensitivity to glucose-induced β-cell apoptosis and negatively to baseline proliferation. In rat islets, constitutive expression of Fas-ligand (FasL) and glucose-induced Fas receptor expression were observed only in older islets. Moreover, PDX-1 expression decreased with age in pancreatic tissue sections of rats and humans. Furthermore, older rat islets were more sensitive to the high glucose mediated decrease in PDX-1 expression than younger islets. Although type-2 diabetes has been accepted and known to be a 'maturity' onset disease, this was thought to be due to genetic predisposition or lifestyle. Our research has shed light on the issue by implicating the disappearance of PDX-1 and FLIP which could now be used as possible targets for treatment of the disease.

We have also implicated the Fas receptor in the development of insulin resistance. Exposing Fas-deficient mice to a high fat diet, surprisingly resulted in no beta-cell impairment, no insulin resistance compared to wildtype mice on the high fat diet, improved PDX-1 and insulin mRNA as well as improved glucose stimulated insulin release, compared to Fas-deficient mice on normal chow.

The Fas/FLIP pathway, therefore, may play an important role in type 2 diabetes. To further elucidate this role, we generated β-cell-specific-FLIP overexpressing mice. These mice had increased β-cell mass, increased insulin mRNA, and improved fasting glucose as the mice aged. Finally, the islets displayed immune cell infiltration, probably due to the induction of IL-8, a chemokine implicated in the attraction of immune cells but also in increased angiogenesis and tumour growth.

Inflammation has always been linked with type 1 diabetes, yet our results show a clear role of cytokines in the impairment of β-cell function as well as in destruction of β-cell mass in a type 2 diabetic milieu. The novelty of this idea has opened up the field of type 2 diabetes to new treatment strategies. A possible treatment would be the use of Interleukin-1 Receptor Antagonist (IL1-Ra), which blocks the action of IL-1β; upregulating FLIP expression in the β-cells to increase β-cell mass and function; or partially blocking the Fas receptor in the β-cell or peripheral tissue thus preventing insulin resistance but the increased beta-oxidation due to the high fat diet would increase β-cell ATP levels and thus increase insulin secretion.

# Zusammenfassung

Die pankreatische β-Zellmasse und Funktion wird bei erhöhtem Insulinbedarf wie bei der Adipositas heraufreguliert. Das Versagen dieser Anpassung resultiert in der Manifestation des Diabetes Mellitus. Die molekularen Mechanismen, welche diesen adaptiven Prozess steuern, sind noch unklar. Dabei ist Fas, ein „death Receptor“, von Bedeutung. Er kontrolliert die β-Zellapoptose oder Proliferation, je nach Aktivität des caspase-8 Inhibitor FLIP. Wir zeigen hier, dass die Fas Signalkaskade auch die sekretorische β-Zellfunktion reguliert. So beobachteten wir eine beeintrchtige Glukosetoleranz in den Fas-defizienten Mäusen. Dies zeigte sich anhand eines verzögerten und verminderten Insulinsekretionsmusters. Von Bedeutung ist die in Fas-defizienten β-Zellen verminderte Expression von PDX-1, ein β-Zellspezifischer Transkriptionsfaktor, welcher die Insulinproduktion und mitochondriale Funktion steuert. Als Konsequenz war die Insulin und ATP-Produktion schwerstgradig reduziert und daher teilweise durch eine dramatische erhöhte β-Zellmasse kompensiert. Aufregulation von FLIP verstärkte die NF$\kappa$B Aktivität via NF$\kappa$B-induzierende Kinase und RelB. Dies führte zu einer Erhöhung der PDX-1 und Insulinproduktion unabhängig vom Zellumsatz.

Hohe Glukosekonzentrationen haben einen dualen Effekt auf den β-Zellumsatz. Kurzzeitig wird die Proliferation induziert, längerfristig jedoch die Apoptose aufreguliert. Die β-Zellen produzieren durch die Hyperglykämie IL-1β, welches das Fas reguliert. Hier wird gezeigt, dass IL-1β in niedrigen Konzentrationen an der mitogenen Wirkung der Glukose via Fas-FLIP Kaskade beteiligt sein könnte. Daher, nach Exposition humaner Inseln mit niedriger IL-1β Konzentrationen (0.01–0.02 ng/ml), wurde eine Stimulierung der Proliferation und einen Minderung der Apoptose registriert. Im Gegensatz dazu hatten höhere Konzentrationen von IL-1β (2–5 ng/ml) den gegenteiligen Effekt. Derselbe bimodale Effekt wurde auch in der Induktion der FLIP, PDX-1 und Pax4 mRNA Expression wie auch bei der Glukose-stimulierte Insulinsekretion verzeichnet. Dagegen war die Induktion des IL-1β monophasisch. Zudem induzierten niedrige IL-1β Konzentrationen den IL-1 Rezeptor Antagonist (IL-1Ra). Wurde der IL-1Ra durch RNA-Interferenz unterdrückt, hoben sich die günstigen Effekte von niedriger IL-1β Konzentration wieder auf. Der Fas-antagonistische Antikörper ZB4 und die FLIP siRNA verhinderten die durch niedrige IL-1β Konzentrationen stimulierte β-Zellproliferation. In Übereinstimmung mit unseren „in vitro“-Experimenten, zeigten die „IL-1β-knockout“-Mäuse eine Glukoseintoleranz sowie Erniedrigung in den Fas, FLIP, Pax4 und PDX-1 Transkripten der Inseln. Diese Erkenntnisse weisen darauf hin, dass niedrige IL-1β Levels die β-Zellfunktion und Turnover mittels Fas-FLIP Kaskade positiv beeinflussen. Zudem zeigt dies auch, dass die IL-1Ra Produktion den schädlichen Effekt von hoher IL-1β Konzentrationen

verhindert.

Der Diabetes Typ 2 ist charakterisiert durch ein Defizit in β-Zellmasse und sein vermehrtes Auftreten in fortschreitendem Alter. Wir analysierten den β-Zellumsatz der Inseln von Ratten verschiedenen Alters (2–3 Monate vs. 7–8 Monate) und von Organspendern mit einer Altersspanne von 17 bis 74 Jahre. Die kultivierten Inseln der 2–3 Monate alten Ratten zeigten bei einer Erhöhung der Glukosekonzentration von 5.5 auf 11.1 mM eine verminderte β-Zellapoptose, welche jedoch bei Steigerung der Glukosekonzentration auf 33.3 mM zunimmt. Parallel dazu konnte sowohl bei 11.1 als auch bei 33.3 mM Glukose eine erhöhte Proliferation, verglichen mit 5.5 mM Glukose, verzeichnet werden. Im Gegensatz dazu beobachteten wir bei den 7–8 Monate alten Ratten und erwachsenen Menschen eine lineare Zunahme des β-Zelltodes und Abnahme der Proliferation bei steigender Glukosekonzentrationen (5.5 bis 33.3 mM). Zusätzlich korrelierte bei kultivierten humanen Inseln das Alter positiv mit der Empfänglichkeit für glukoseinduzierte β-Zellapoptose und negativ mit der Basisproliferation. Die Inseln der 7–8 Monate alten Ratten zeigten im Gegensatz zu den der 2–3 Monate alten Ratten eine konstitutive Expression von Fas-Ligand (FasL) und des glukoseinduzierten Fas-Rezeptors. Darüber hinaus wurde in den pankreatischen Gewebsschnitten von Mensch und Ratte eine mit zunehmendem Alter verminderte PDX-1 Expression nachgewiesen. Zusätzlich sind die älteren Ratteninseln anfälliger auf die, durch eine hohe Glukosekonzentration vermittelte, Erniedrigung von PDX-1. Somit könnte man die Unterschiede in der Glukosesensitivität zwischen den humanen Inseln und den Inseln der 2–3 Monate alten Ratten vielmehr auf altersabhängige Veränderungen als auf den genetischen Hintergrund zurückführen. Diese Daten liefern eine mögliche Erklärung für das vermehrte Auftreten des Diabetes Typs 2 im fortschreitendem Alter. Auerdem weisen sie darauf hin, dass Inseln älterer Ratten ein passenderes Model für die Erforschung der glukoseinduzierte β-Zellapoptose sind.

Wir etablierten ein „high-fat“ induziertes diabetisches Tiermodell. Diese Mäuse entwickelten nach 4-wöchiger „high-fat“ Diät eine beeinträchtigte Glukosetoleranz und Insulinresistenz. Überraschende Resultate ergaben sich durch die Exposition der Fas-defizienten Mäuse mit dieser Diät. Gegenüber den Fas-defizienten Mäusen mit normaler „chow“ Diät verbesserte sich die Glukosetoleranz und normalisierte sich die erste und zweite Phase der Insulinsekretion. Zudem entstand bei den Fas-defizienten Mäusen im Vergleich zu den Wiltypen-Mäusen mit „high-fat“ Diät auch keine Insulinresistenz. Die Analyse der Inseln von den unter der „high-fat“ Diät gehaltenen Fas-defizienten Mäusen ergab eine Erhöhung in den Insulin und PDX-1 mRNA Levels sowie eine Verbesserung der glukosestimulierten Insulinsekretion. Um die „in vivo“-Situation nachzuahmen, führten wir „in vitro“-Experimente durch, bei denen Fas-defiziente Inseln einer Mixtur von Palmitat und Oleat ausgesetzt wurden. Die, mit Fettsäuren behandelten, Fas-defizienten Inseln zeigten ebenfalls eine Verbesserung der glukosestimulierten Insulinsekretion gegenüber den unbehandelten Fas-defizienten Inseln, wobei die Wildtypen-Inseln nach derselben Fettsäure-Exposition eine verminderte Funktion aufwiesen. Ebenso zeigte sich, im Gegensatz zu den Wildtypen-Inseln, keine Beeinträchtigung der glukosestimulierten Insulinsekretion nach Exposition der Fas-defizienten Inseln mit chronischer Hyperglykämie. Es gibt drei mögliche Theorien, welche die verbesserte Insulinsekretion der Fas-

defizienten Maus nach Behandlung mit „high-fat“ Diät oder der Fas-defizienten Inseln nach Fettsäure-Exposition erklären könnten: 1) Schutz der Fas-defizienten Inseln gegenüber der Fettsäureexposition. 2) Wiederaufnahme der Insulinsekretion via ATP-Produktion durch β-Oxidation der Fettsäure. 3) Unfähigkeit der Fas-defizienten Mäuse eine Insulinresistenz zu entwickeln.

Die Fas-FLIP Kaskade scheint daher eine wichtige Rolle im Diabetes Typ 2 zu spielen. Um diese Rolle weiter zu erforschen, generierten wir Mäuse, welche β-Zell-spezifisch FLIP überexprimieren. Diese Mäuse hatten im Alter eine gesteigerte β-Zellmasse, erhöhte Insulin mRNA und verbesserte Nüchternglukose. Ebenso zeigten die Inseln ein Infiltrat von Immunzellen, wahrscheinlich aufgrund der Induktion von IL-8, ein Chemokin, welches chemoattraktiv auf neutrophile Granulozyten wirkt und in Zusammenhang mit der Angiogenese und Tumorwachstum gebracht wird.

Somit scheint die Fas-FLIP Kaskade eine wichtige Rolle in der Insulinsynthese und Sekretion zu spielen.

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# 1 Introduction

## 1.1 Pancreas

The pancreas consists of exocrine and endocrine cells which produce pancreatic digestive enzymes and endocrine hormones, respectively. The bulk of the pancreas consists of exocrine cells, called acini, and embedded in the exocrine tissue are the endocrine cells, called the Islets of Langerhans, which form approximately 1% of the total mass of the pancreas. The pancreatic digestive enzymes, secreted by the exocrine pancreas, are secreted into the intestine where they digest proteins, fats and carbohydrates.

Although the Islets of Langerhans form such a small part of the pancreas, the function of the cells are vastly important to the homeostasis of the body. The Islets of Langerhans consist of the following cell types:

- $\beta$-cells secrete insulin (60–80%)
- $\alpha$-cells secrete glucagon (10–20%)
- $\delta$-cells secrete somatostatin ( 5%)
- PP-cells contain pancreatic polypeptide ($< 1\%$)

The cytoarchitecture of the rodent islet is the $\alpha$ cells found on the periphery, encircling the $\beta$-cells and the interspersed $\delta$ and PP cells (Figure 1.1). The hormones secreted by these cells have an important role in regulating blood glucose in the fed and fasted state. Insulin and glucagon have opposite effects on blood glucose levels. When blood glucose levels are high (e. g. after a meal), the $\beta$-cell produces more insulin which acts on different cells, including muscle, liver and fat cells, thus increasing the uptake of glucose into the cells and returning circulating blood glucose to normal. The $\alpha$ cells secrete glucagon when blood glucose levels are low e. g. after exercise or in-between meals. Glucagon mainly acts on the liver causing gluconeogenesis and the release of stored glucose into the bloodstream. Somatostatin is secreted in response to high levels of the other pancreatic endocrine hormones and thus regulates the production of these hormones. Pancreatic polypeptide (PP) is released in response to a meal ingestion and has been shown to affect the secretion of pancreatic enzymes, gastric emptying and gallbladder motility and appears to be a modulator of peripheral insulin action.

There are many diseases of the pancreas, but in this thesis we are mainly focussing on Diabetes.

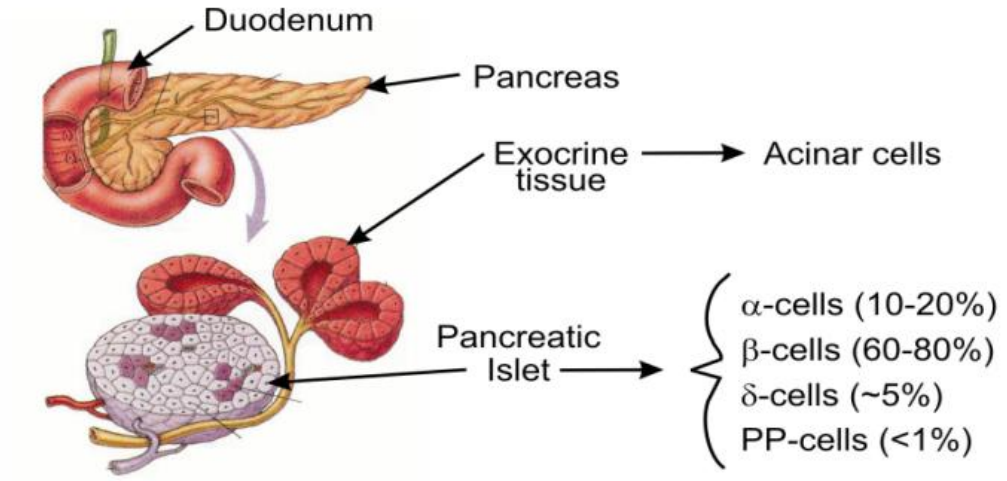


**Figure 1.1:** The structure of a pancreas.

## 1.2 Diabetes

With lifestyles becoming more sedentary, the incidence of obesity has increased and so has the incidence of type 2 diabetes, so much so that the terms obesity and type 2 diabetes are becoming synonymous. Many obese individuals, however, only develop insulin resistance, as the β-cell is able to compensate by increasing in functional β-cell mass. In those individuals where diabetes develops, insufficient compensation occurs which leads to a relative insulin deficiency, glucose intolerance and therefore increased circulating glucose concentrations or hyperglycaemia. Once hyperglycaemia is present, the loss of β-cell mass accelerates accompanied by impairment of the β-cell secretory function, both factors of which are needed for the manifestation of type 2 diabetes. The preservation of a functional β-cell mass and the elucidation of the decline in β-cell function, therefore, have become the focal point of research in diabetes.

### 1.2.1 Glucose and the IL-1β-Fas-FLIP pathway

Under normal conditions, insulin (Figure 1.2) secretion is tightly regulated by the metabolic requirements of the tissues and the circulating glucose concentrations. The breakdown of glucose results in an increase in adenosine triphosphate (ATP), which increases the ATP/ADP ratio (Figure 1.3). The change in ratio closes the ATP-dependent potassium channel ($K^+$ATP channel) in the cell membrane, which in turn depolarizes the cell membrane. The calcium ($Ca^{2+}$) channels open, resulting in an influx of $Ca^{2+}$ into the cytoplasm and insulin exocytosis [2]. Insulin secretion is phasic, with a first phase of secretion which has a duration of less than 10 min, followed by a longer second phase secretion. This is probably due to the existence of

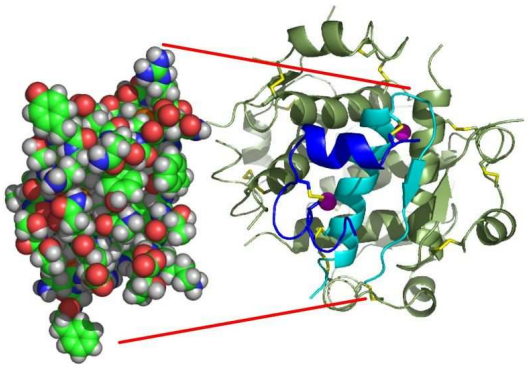

**Figure 1.2:** Left: Carbon is denoted in green, Hydrogen in white, Oxygen in red and Nitrogen in blue. Right: An insulin monomer with the A chain coloured in blue,and the B chain in cyan. Yellow denotes the disulphide bonds and magenta the zinc ions. Adapted from Chang et al. [1].

two pools of insulin granules, with the first phase secretion developing from a readily available pool primed for secretion [2]. Once released, insulin binds to insulin receptors which are expressed ubiquitously, leading to modifications of a number of biological processes, such as cell survival, glucose transport and lipid metabolism. When insulin binds to its receptor, it activates tyrosine kinase, autophosphorylating tyrosine residues on the receptor β-subunit which in turn phosphorylates the insulin receptor substrate proteins (IRS) [3]. The biological process activated depends on which of the IRS proteins are phosphorylated. IRS-1 is mainly involved in cell growth and insulin action in the muscle and adipose tissue. IRS-2 is important for β-cell survival and growth, amongst other things[4]. These functions depend on the recruitment and activation of any of the various downstream signalling molecules, such as phosphatidylinositol 3-kinase (PI 3-kinase), growth factor receptor binding protein 2 (Grb-2) and SH2-containing protein-tyrosine phosphatase-2 (SHP-2)

In cultured human islets, glucose has been shown to induce β-cell proliferation in the short-term but chronically, induced β-cell apoptosis. This duality in function centres around a cell surface receptor called Fas (CD95). Fas belongs to the TNF receptor superfamily and is a transmembrane protein with three extracellular cysteine-rich domains [5]. There are 4 steps to the initial signalling events of the Fas receptor: trimerization, recruitment, surface clusters and internalization. Binding of Fas ligand (FasL) to Fas, causes trimerization of the Fas receptor and recruitment of the adaptor molecule, Fas-associated death domain protein (FADD) and procaspase-8 into the death-inducing signaling complex (DISC) (Figure 1.4). Caspases are a family of aspartate-specific cysteine proteases that are synthesised as proenzymes, activated by proteolytic activity and are required for apoptosis [6, 7]. Procaspase-8 binds to FADD through its N-terminal death effector domain (DED) [8]. Procaspase-8 cleavage occurs via 2 steps: initial cleavage generates a p43 and p12 fragment further processed to a p10 fragment. Subsequent cleavage of the receptor-bound p43 results in formation of the prodomain p26 and the release of the active fragment p18 [8]. Cleavage occurs via a trans-and autocatalytic cleavage due to the close proximity of

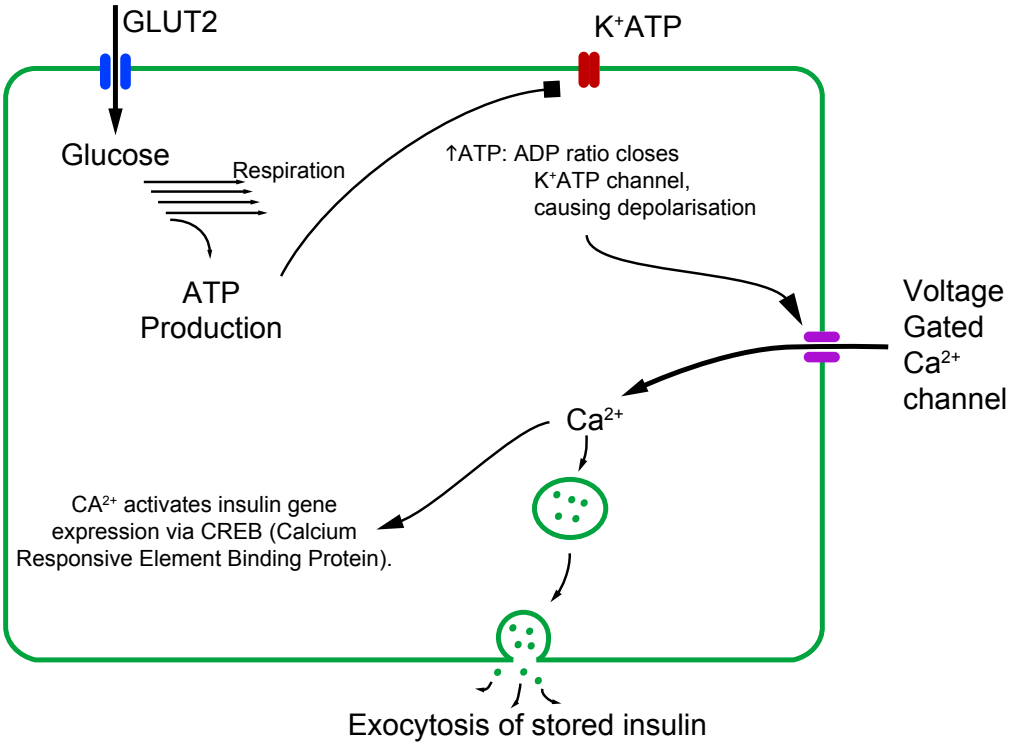


**Figure 1.3:** Schematic of glucose-induced insulin secretion.

the procaspase-8 molecules to each other [6] Prolonged activation of Fas results in all procaspase-8 being activated to caspase-8. Caspase-8 then activates other caspases further downstream, setting a cascade in motion with the end result being apoptosis.

Since exposure of β-cells to glucose resulted in an increase in proliferation in the short-term and since not all activation of the Fas receptor resulted in apoptosis, it became clear that an inhibitor or anti-apoptotic molecule must exist in the Fas pathway. At first, this molecule was known by many names, FLAME-1, CASPER, FLIP, Usurpin, CLARP, I-FLICE, MRIT, as it was discovered independently in many groups [10–13]. FLIP is an unstable protein which is rapidly degraded via the ubiquitin-proteosome pathway. FLIP is predominantly expressed in the heart and lymphoid tissue but is also expressed in the kidneys and pancreas [10]. NF$\kappa$B, protein synthesis inhibitors, calcium/calmodulin-dependent protein kinase II, bile acids, heat stress, transforming growth factor beta, oxidised LDL, and p53 are possible regulators of FLIP expression [6, 14–21]. Two splice variants of FLIP exist, called FLIP-Short( $\text{FLIP}_\text{S}$) and FLIP-Long ($\text{FLIP}_\text{L}$). FLIP has a similar structure to caspase-8 though $\text{FLIP}_\text{S}$ only contains 2 death effector domains and $\text{FLIP}_\text{L}$ contains 2 death effector domains and an additional inactive caspase-like domain. Both $\text{FLIP}_\text{S}$ and $\text{FLIP}_\text{L}$ are recruited to FADD and block activation of procaspase-8 to caspase-8, thus inhibiting apoptosis. $\text{FLIP}_\text{S}$ completely blocks any processing of procaspase-8 as it contains no caspase-like domain, whereas $\text{FLIP}_\text{L}$ allows the first cleavage step of procaspase-8 to p10, but further processing is inhibited [7, 22].

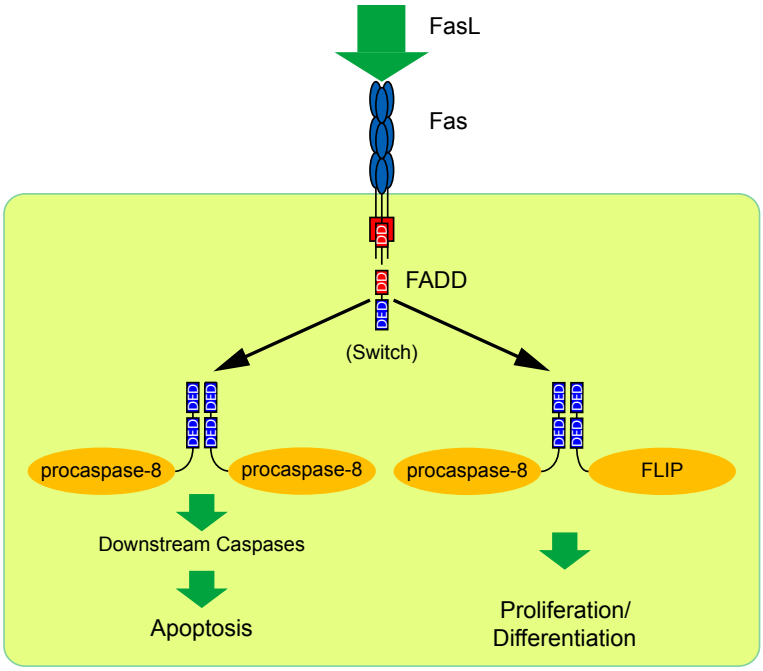


**Figure 1.4:** The Fas pathway, which depending on the presence of FLIP can be apoptotic or proliferative. Adapted from Budd [9].

When insulin signalling is disrupted or insulin resistance occurs, circulating glucose concentrations increase resulting in chronic hyperglycaemia. Chronic hyperglycaemia has severe adverse effects on β-cell function causing glucose desensitization, β-cell exhaustion and finally the irreversible reduction of insulin secretion as well as insulin stores. This is termed glucotoxicity. Chronic hyperglycaemia induces Fas expression via the cytokine IL-1β. Fas expression induced by cytokines in the β-cell requires the activation of NF$\kappa$B. Nuclear Factor kappa B (NF$\kappa$B) does not refer to one molecule but rather to a group of proteins (RelA, RelB, c-Rel, p105/p50 and p100/p52) which share a conserved N-terminal region called a Rel homology (RH) domain [23]. This RH domain contains the DNA-binding and dimerization domains and the nuclear localization signal (NLS) which enables the regulation of the nuclear-cytoplasmic shuttling of NF$\kappa$B [24]. There are two main NF$\kappa$B signalling pathways:

- classical or canonical pathway,
- alternative or non-canonical pathway.

### Classical / Canonical pathway

NF$\kappa$B is activated by various extracellular stimuli such as Tumor necrosis factor alpha (TNF$\alpha$), interleukin 1-beta (IL1-β), and Fas (Figure 1.5). When the canonical pathway is activated, the IkappaB (I$\kappa$B) kinase (IKK) complex which consists of two kinase subunits, IKK$\alpha$ and IKKβ and a structural subunit IKK$\gamma$ or NEMO (NF$\kappa$B essential modulator), phosphorylates I$\kappa$B$\alpha$ [23–25]. I$\kappa$B is attached to a heterodimer

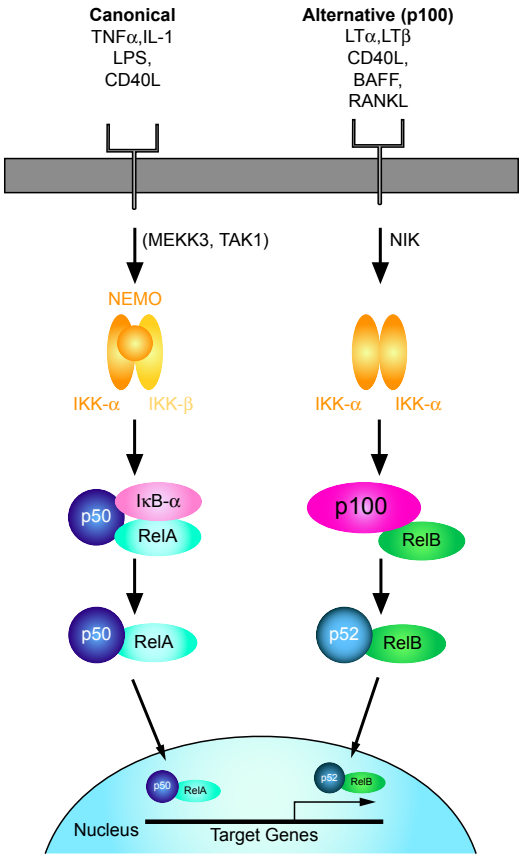


**Figure 1.5:** NF$\kappa$B has two main pathways, canonical and alternative. Adapted from Beinke and Ley [25].

consisting of p50 and RelA (also called p65). I$\kappa$B's, the NF$\kappa$B inhibitors, sterically block the function of the nuclear localization signal of the NF$\kappa$B dimers thus preventing nuclear translocation [23]. Immediately after phosphorylation, the I$\kappa$B$\alpha$ is ubiquitinated and soon thereafter degraded by the 26S proteosome. The degradation of I$\kappa$B$\alpha$ frees the p50/RelA heterodimer, which translocates into the nucleus, binds to the promoter regions of NF$\kappa$B-responsive genes such as IL1-$\beta$ and interleukin-6 (IL6), and modulates their expression [25].

### Alternative / non-canonical pathway

Activation of the alternative pathway results in phosphorylation of the IKK$\alpha$ homodimer and thus activation of its kinase activity by NF$\kappa$B-inducing kinase (NIK) (Figure 1.5). A mutation in NIK, as occurs in the alymphoplasia (aly/aly) mice, inhibits further processing of this pathway. The phosphorylated IKK$\alpha$ homodimer phosphorylates p100 which is bound to RelB. p100 also functions as an I$\kappa$B as it has an ankyrin repeat region in the C-terminal. After phosphorylation, p100 is slowly processed by proteolysis to form p52, which together with RelB translocates to the nucleus.

The NF$\kappa$B pathway has mainly been linked with the regulation of immune and

inflammatory responses, but more recent evidence has linked it with cell proliferation, apoptosis and insulin secretion [5, 19]. Although FLIP is regulated by NF$\kappa$B, evidence indicates that NF$\kappa$B is in-turn regulated by FLIP [19, 26–29].

### 1.2.2 Age and Diabetes susceptibility

The fact that type 2 diabetes predominantly occurs in older individuals is an accepted fact, especially as this disease is known as 'adult onset diabetes'. Although human islets constitutively express FasL, human islets from older organ donors are more susceptible to glucose-induced apoptosis. It is a well known fact that glucose is the principle regulator of insulin biosynthesis, both at the transcriptional and post-transcriptional levels via the pancreatic duodenal homeobox (PDX-1) [30–33]. PDX-1 is a transcription factor expressed in β-cells, some $\delta$ cells, in dispersed endocrine cells of the duodenum and in the developing brain [32]. As seen in homozygous PDX-1 knockout mice, PDX-1 is important for the development of the pancreas [34]. In the adult mouse, however, PDX-1 mainly regulates insulin gene expression, but also plays a role in glucose transporter 2 (GLUT-2), glucokinase, and islet amyloid polypeptide regulation [35]. Prolonged exposure of human, mouse, or rat islets to high glucose results in a decrease in PDX-1 mRNA as well as a decrease in insulin mRNA, resulting in impaired function of the β-cell [33, 36–42]. Marshak et al. [37] found that chronic exposure to high glucose in human islets lowers PDX-1 binding activity. The difference in function of PDX-1 from pancreatic development to insulin gene expression in adulthood, made us question its role in the development of diabetes with age.

### 1.2.3 Free fatty acids and Diabetes

Free fatty acids (FFA) are elevated in obese individuals and have been implicated in the loss of insulin sensitivity and thus the onset of insulin resistance [43]. Lowering free fatty acid concentrations in diabetic individuals, resulted in normalized insulin sensitivity [44]. Free fatty acids don't only affect the peripheral tissues (skeletal muscle and liver), but also directly affect the β-cells. FFAs acutely stimulate insulin secretion but chronic exposure of islets to FFAs results in desensitisation and suppression of insulin secretion [45]. In the Zucker Diabetic Fatty (ZDF) rat it has been found that high circulating FFAs and triglyceride levels induce triglyceride accumulation in islets [46]. This increase in triglyceride levels causes β-cell apoptosis via the ceramide pathway. Not all obese individuals or type 2 diabetes susceptible individuals exhibit dyslipidemia. It is therefore possible that though lipotoxicity plays an important role in β-cell destruction, the situation is probably worsened in the presence of high glucose concentrations, thus leading to the phenomenon, glucolipotoxicity.

Since NF$\kappa$B which is needed for insulin secretion is found downstream of both IL-1β and Fas, we hypothesised that both IL-1β and Fas are needed for β-cell function and insulin secretion.

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# 2 The Fas pathway is involved in β-cell secretory function

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**Abstract**

Pancreatic β-cell mass and function increase in conditions of enhanced insulin demand such as obesity. Failure to adapt leads to diabetes. The molecular mechanisms controlling this adaptive process are unclear. Fas is a death receptor involved in β-cell apoptosis or proliferation, depending on the activity of the caspase-8 inhibitor FLIP. Here we show that the Fas pathway also regulates β-cell secretory function. We observed impaired glucose tolerance in Fas deficient mice due to a delayed and decreased insulin secretory pattern. Expression of PDX-1, a β-cell specific transcription factor regulating insulin production and mitochondrial metabolism, was decreased in Fas deficient β-cells. As a consequence, insulin and ATP production were severely reduced and partly compensated for by a dramatic increase in β-cell mass. Up-regulation of FLIP enhanced NF-$\kappa$B activity via NF-kappaB-inducing kinase and RelB. This led to increased PDX-1 and insulin production independent of changes in cell turnover. The results support a role for the Fas-pathway in regulating insulin production and release.

## 2.1 Introduction

A decrease in functional insulin-producing pancreatic β-cells contributes to the establishment of not only type 1 but also of type 2 diabetes [1–3]. Fas (CD95) is a cell surface receptor which plays a central role in the regulation of death in many cell types including β-cells and may be implicated in the development of type 1 and 2 diabetes [4–6]. Glucose-induced β-cell apoptosis in human islets involves IL-1β mediated up-regulation of Fas and subsequent interaction with the constitutively expressed Fas ligand (FasL) on neighboring β-cells [7–10]. Fas/FasL interaction leads to cleavage of procaspase-8, the most upstream caspase in the Fas apoptotic pathway. However, elevated glucose concentrations have a dual effect on β-cell turnover inducing proliferation in the short-term and apoptosis in the long-term [7, 11, 12]. This dual effect of glucose may be explained by the pivotal role of FLICE-inhibitory protein (FLIP) on Fas signaling. FLIP structurally resembles caspase-8 but lacks its

proteolytic activity [13]. FLIP is expressed in human pancreatic β-cells, and is decreased in the pancreatic tissue of type 2 diabetic patients as well as in cultured islets following prolonged exposure to elevated glucose concentrations [14]. Upregulation of FLIP switches Fas-mediated glucose signaling in β-cells from being apoptotic to favoring cell replication [14].
The transcription factor nuclear factor-$\kappa$B (NF-$\kappa$B) is an essential component of cytokine signaling. Interestingly, in β-cells, NF-$\kappa$B activation can also be triggered by glucose [8, 15]. Increasing evidence points to non-inflammatory effects of NF-$\kappa$B, including regulation of insulin secretion [16]. NF-$\kappa$B is activated by signaling through many receptors, which can be grouped into the classical pathway and the non-classical pathway of NF-$\kappa$B activation [17]. In the classical pathway, upstream signals induce phosphorylation of I$\kappa$B$\alpha$ bound to cytosolic NF-$\kappa$B. Phosphorylation is carried out by the I$\kappa$B kinase (IKK) complex, which is composed of IKK-$\gamma$ and two catalytic subunits, IKK-$\alpha$ and IKK-β. Phosphorylation tags I$\kappa$B$\alpha$ for ubiquitylation and, ultimately, for proteasomal degradation, liberating NF-$\kappa$B (p50 and RelA or p65) for translocation to the nucleus and subsequent activation of target genes. The non-classical pathway is controlled through NF-$\kappa$B-inducing kinase (NIK) which phosphorylates IKK-$\alpha$; this regulation occurs independently of the classical IKK complex and leads to processing of p100, generating p52-RelB heterodimers, which migrate to the nucleus.
The β-cell specific transcription factor PDX-1 predominantly regulates β-cell differentiation and secretory function. In particular, PDX-1 controls several events in glucose stimulated insulin secretion including mitochondrial metabolism [18–21]. Several observations point to a role for the Fas pathway in the regulation of cell-cycle independent events including T cell activation [22], renal tubular epithelial integrin function [23], and protection against neurodegeneration [24]. However, a direct interaction between the Fas pathway and PDX-1 has not been investigated.
In addition to its effect on β-cell turnover, hyperglycemia impairs β-cell secretory function [2, 25–27]. This glucotoxic effect is evident before apoptosis leads to a significant decrease in β-cell mass. This is most striking in vitro, where a 4-day exposure of human islets to elevated glucose concentrations leads to almost complete ablation of β-cell secretory function although less than 1% of β-cells are apoptotic [8]. Since hyperglycemia regulates Fas expression [7, 9], we hypothesize that the Fas pathway may not solely mediate glucose-induced changes in cell-turnover, but also changes in β-cell secretory function.

## 2.2 Experimental Procedures

### 2.2.1 Animals

Ethical approval for mouse studies was granted by the Zürich Cantonal Animal Experimentation Committee. C57BL/6j wildtype and mice with a natural Fas mutation backcrossed for more than ten generations onto this same C57BL/6j inbred strain background (B6.MRL$^{lpr}$) were obtained from The Jackson Laboratory (Bar Harbor, ME). NODβFas$^{-/-}$ and BL6βFas$^{-/-}$ mice were produced by introduction of LoxP

sites flanking exon IX of Fas and breeding to NOD and C57Bl/6 rat insulin promoter (RIP)-Cre mice (A. V. C. et al., manuscript in preparation). The aly/aly (alymphoplasia or Map3k14$^{aly}$) mice which carry a point mutation in the NF$\kappa$B-inducing kinase (NIK, encodec by Map3k14) were bred in-house [28]. Animals were housed at 22°C with a 12-h light-dark cycle (lights on at 07:00) and allowed free access to water and chow.

### 2.2.2 Intra-peritoneal glucose and insulin tolerance tests

Mice were fasted 12 h overnight and injected intra-peritoneally with 1 or 2 mg/g body weight glucose (40% glucose solution, Laboratorium Dr. G. Bichsel AG, Interlaken, Switzerland) or with 0.5 mU/g recombinant human insulin (Novo Nordisk A/S, Bagsvrd, Denmark) for the glucose or insulin tolerance test, respectively. Blood samples were obtained from tail-tip bleedings, and blood glucose concentrations were measured with a Glucometer (Freestyle, Disetronic Med. Systems, Burgdorf, Switzerland).

### 2.2.3 In situ pancreas perfusion

Mice starved overnight were anesthetized with sodium pentothal intra-peritoneally and prepared for pancreas perfusion as previously described [29]. The pancreas was perfused at 37°C with 1.5 ml/min of modified Krebs-Ringer HEPES buffer supplemented with 2.8 mM glucose (basal) and with 16.7 mM glucose for stimulation. The pancreatic effluent of the first 30 min of perfusion with basal glucose was not collected. After this equilibration period, the effluent was collected in 1 min fractions from a catheter placed in the portal vein. The insulin content of each fraction was determined by radioimmunoassay [30].

### 2.2.4 Islet cell isolation and culture

Human islets were isolated from pancreata of organ donors at the University of Geneva Medical Center and mouse islets were isolated as previously described [31]. The islets were cultured on extracellular matrix-coated plates derived from bovine corneal endothelial cells (Novamed Ltd., Jerusalem, Israel), allowing the cells to attach to the dishes and spread into a monolayer. Mouse islets were cultured in RPMI 1640 medium containing 11.1 mM glucose, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 40 $\mu$g/ml gentamycin and 10% FCS (Invitrogen Ltd., Carlsbad, CA), and human islets in CMRL 1066 medium containing 5.5 mM glucose, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 10% FCS, hereafter referred to as culture medium. In some experiments, one day after plating the islets, the medium was changed to culture medium containing 500 ng/ml mouse antagonistic anti-Fas antibody (ZB4; MBL, Nogoya, Japan), or 500 ng/ml mouse IgG isotype control (R&D Systems Inc.). The initial characterisation of ZB4 was performed in Jurkat cells [32]and was shown to be efficient in different human cell types including lymphocytes [33], endothelial cells [34], and β-cell [7]. The INS1E cell line was cultured

in RPMI 1640 containing 11.1 mM glucose, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 40 $\mu$g/ml gentamycin and 10% FCS and beta mercaptoethanol.

### 2.2.5 Liposome-mediated transfection

Lipofectamine 2000-DNA complexes were prepared according to the manufacturer's instructions (Invitrogen Ltd.) using a RIP-FLAG-tagged-FLIP-long- (generously provided by J. Tschopp, University of Lausanne, Switzerland) or RIP-plasmid-DNA (control) or a CMV-Fas-plasmid-DNA (generously provided by S. Nagata, Osaka University Medical School, Japan). The solution was added to the islets and INS1E cell line (70% confluency) at a final concentration of 3 $\mu$g of DNA/ml. After 6 h incubation, 2 ml culture medium was added to the islets, and after 24 h the medium was aspirated and replaced with fresh culture medium. For the INS1E cell line, the media was aspirated and replaced with fresh culture media after the 6 h incubation period.

### 2.2.6 Immunostaining and histochemical analysis

The entire pancreas was rapidly resected from euthanized mice, all fat and non-pancreatic tissue trimmed and the pancreas was weighed. The mean weight of the pancreata did not differ between groups at any age. A longitudinal section of the pancreas (tail through head in the flat plane of the pancreas) was fixed in formaldehyde adn then embedded in paraffin. Sections of pancreas were then taken through the fixed tissue in the plane of embedding so that a near complete section of pancreas (head, body and tail) through its maximal width was obtained with each section. For insulin and glucagon double-labeling, paraffin embedded tissue sections were incubated with guinea pig anti-insulin antibody (Dako, Carpinteria, CA) followed by detection with a fluorescein-conjugated rabbit anti-guinea pig antibody (Dako). Subsequently, the specimens were labeled for glucagon with mouse anti-glucagon antibody (Dako), followed by detection with donkey anti-mouse Cy3-conjugated antibody (Jackson ImmunoResearch Laboratories West Grove, PA). In digital images of individual islets, the area of insulin immunopositive cells was traced manually and computed using analySISTM 3.1 software (Soft Imaging System GmbH, Muenster, Germany). The $\beta$-cell volume of each islet was estimated by using $\beta$-cell area and by assuming spherical islets. After washing with PBS, cultured islets were fixed in 4% paraformaldehyde followed by permeabilisation with 0.5% triton X-100 and incubation with mouse anti-Fas antibody (Transduction Laboratories, Lexington, KY). Detection was performed using the streptavidin-biotin-peroxidase complex (Zymed).

### 2.2.7 Anti-nuclear antibodies

Anti-nuclear antibodies were detected by incubation of a mouse liver tissue section with test serum followed by detection with anti-mouse Cy3-conjugated antibody (Jackson). Serum of wildtype and of diseased mice was used as negative and positive controls, respectively.

### 2.2.8 Cytokine assay

Serum IL-1β, IFN$\gamma$, IL-6 and TNF$\alpha$ were measured using Luminex technology according to the manufacturer's instructions (Labodia, Préverenges, Switzerland).

### 2.2.9 Cell replication and apoptosis

For β-cell replication and apoptosis studies, islets were double stained with either a monoclonal antibody against the mouse Ki-67 antigen (Zymed, San Francisco, CA) or by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) according to the manufacturer's instructions (In Situ Cell Death Detection Kit, AP; Boehringer Mannheim, Germany) and for insulin as previously described [35].

### 2.2.10 Insulin release, and insulin and ATP content

For acute insulin release in response to glucose, islets were washed and pre-incubated (30 min) in Kreb's Ringer bicarbonate buffer (KRB) containing 2.8 or 3.3 mM glucose and 0.5% BSA. KRB was then replaced by KRB 2.8 or 3.3 mM glucose for 1 h (basal), followed by an additional 1 h in KRB 16.7 mM glucose or 2.8 mM glucose and 20 mM leucine, 10 mM $\alpha$-ketoglutarate dimethyl ester, 10 mM succinic acid dimethyl ester or a mixture of 10 $\mu$M forskolin and 100 $\mu$M 3-isobutyl-1-methylxanthine (Sigma). Islets were extracted with 0.18 N HCl in 70% ethanol for determination of insulin content. To determine total insulin content of the pancreas, the tissue was homogenized in 1 ml 0.18 N HCl in 70% ethanol and left overnight at 4°C. Insulin was determined using a human insulin RIA kit (CIS bio international, Gif-Sur-Yvette, France), which has similar affinity for both mouse and human insulin. For determination of islet ATP content, islets were cultured overnight and preincubated for 30 min in KRB followed by a 10 min stimulation with 16.7 mM glucose KRB. Islet-ATP content was quantified with a Bioluminescence-based kit (Roche).

### 2.2.11 RNA extraction and quantitative reverse transcription-PCR (RT-PCR)

We extracted total RNA from the cultured islets by using the Rneasy mini kit (Qiagen, Basel, Switzerland) and performed RT-PCR by using the SuperScript Double-Stranded cDNA synthesis kit according to the manufacturer's instructions (Life Technologies, Gibco, Gaithersburg, MD). For quantitative analysis, we used the Light Cycler quantitative PCR system (Roche, Basel, Switzerland) with a commercial kit (Light Cycler-DNA Master SYBR Green I; Roche). Mouse primers used were 5′TACGGGGTTTGTGAAAGGAG3′ and 5′CACATCATTCCCCAGGAAAC3′ (insulin); 5′GAGGACCCGTACAGCCTACA3′ and 5′CGTTGTCCCGCTACTACGTT3′ (PDX-1); 5′CTAAATTTGGTTGCCCCAGA3′ and 5′CTCCCATTATGGAGCCTGAA3′ ($FLIP_L$); 5′CAGCCAGCGCCCAGTACC3′ and 5′CAATGCGGACGGAGGCAAAGC3′ (UCP-2); 5′GTGGCAGTGATGGCATGGAC3′ and 5′CAGCACC-

AGTGGATGCAGGG3′ (GAPDH); 5′AGAGTCGCGCTGGTAAGAAGC3′ and 5′CCCCAATGGTCTTGTCACTT3′ (Tubulin). Human primers used were 5′CTCTACAATGGGCTGGTTGC3′ and 5′TTGGTATCTCCGACCACCTC3′ (UCP-2); 5′CCACCTTGGGACCTGTTTAG3′ and 5′TGATGCCAGAGGAAGAGGAG3′ (PDX-1); 5′GCTGGTAGAGGGAGCAGATG3′ and 5′CTCACACCTGGTGGAAGCTC3′ (insulin), 5′AGAGTCGCGCTGTAAGAAGC3′ and 5′TGGTCTTGTCACTTGGCATC3′ (Tubulin); 5′AACAGCGACACCCACTCCTC3′ and 5′GGAGGGGAGATTCAGTGTGGT3′ (GAPDH).

### 2.2.12 Western Blot

Protein was extracted using an extraction buffer containing 20 mM Tris pH 7, 1% Triton-X-100, 150 mM NaCl, and 10% glycerol. The following protease inhibitors were added immediately before use: 200 $\mu$l protease inhibitor cocktail (Roche), 20 $\mu$l Na3VO4, 20 $\mu$l NaF, 10 $\mu$l PMSF and 4 $\mu$l 0.5 M EDTA (pH 8). After extraction, an aliquot was used for protein determination and the remainder was frozen at –80°C in sample buffer. 30 $\mu$g Protein was loaded on a NuPage gel (Invitrogen), blocked for 1 hour in TBS-T containing 5% milk. The membrane was exposed to a rat monoclonal antibody to FLIP (Dave-2, Alexis biochemicals), a rabbit anti-PDX-1 antibody (kindly donated by H. Edlund) and a rabbit anti-Actin antibody (Santa Cruz). The secondary antibody was a goat polyclonal anti-rat IgG HRP (Alexis biochemicals), or a donkey anti-rabbit IgG HRP (Santa Cruz). The emitted light was captured on Kodak Bio Max Film after the membrane was exposed to Lumilight western blotting substrate (Roche).

### 2.2.13 RNA interference

RNAs of 21 nucleotides, designed to target FLIPlong (Silencer Pre-designed siRNA) and scrambled siRNA were synthesized by Ambion (Austin, TE). siRNA was transfected using SiPortAmineTM, and transfection efficiency estimated with cy3 labeled siRNA using SilenceTM siRNA Labeling Kit (Ambion), as described previously [36].

### 2.2.14 NF-$\kappa$B activation

For the gene reporter assay, INS-1E cells were transiently transfected according to the manufacturer's instructions using Superfect (Qiagen, Hilden, Germany) and a total of 1 $\mu$g plasmid DNA. NF-$\kappa$B-dependent gene transcription was analysed with 0.4 $\mu$g of the PathDetect NF$\kappa$B cis-Reporting System (Stratagene, San Diego, CA). The luciferase reporter gene was selectively regulated by 5x synthetic NF-$\kappa$B promoter enhancer elements. In all gene reporter assays, cells were also transfected with an empty vector (pcDNA3, Invitrogen) as control. The promoter activity was analysed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. For detection of specific activation of NF-$\kappa$B complex by the classical pathway and the non-classical pathway nuclear extraction was performed as per the manufacturer's instructions (Nuclear Extract Kit, Active Motif)

followed by analysis with ELISA-based Kits, using attached oligonucleotides binding to a NF-$\kappa$B consensus site and detected by an anti-p65 or p50 subunit antibody and by an anti-Relb subunit antibody, according to the manufacturer's instructions (Trans-AMTM NF$\kappa$B and Trans-AMTM NF$\kappa$B Family, Active Motif).

### 2.2.15 Determination of glucose uptake

Glucose uptake measurements into isolated white adipocytes were carried out as described previously [37]. Briefly, epididymal white adipose tissue was dissected and adipocytes were isolated with collagenase in KRB containing 4% (w/v) fatty acid-free BSA and filtered through a 250 $\mu$m nylon mesh. Adipocytes were washed twice and resuspended in KRB 1% BSA. Cells were incubated for 60 min with or without 100 nM insulin in U-$^{14}$C-glucose (final glucose concentration 1 mM). The reaction was terminated by centrifugation through a layer of dinonylphthalate followed by liquid scintillation counting.

### 2.2.16 Acute glucose stimulated insulin synthesis

For radiolabeling, batches of 120 to 220 islets from a single mouse pancreas were washed in KRB-Hepes, 0.25% BSA (KRB-Hepes-BSA), 2.8 mM glucose and preincubated for 15 min at 37°C in this same buffer. The islets were resuspended in 100 $\mu$l KRB-Hepes-BSA with either 2.8 or 16.7 mM glucose and containing 590 $\mu$Ci/ml [$^3$H]leucine (specific radioactivity 150 Ci/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO). The islets were radiolabeled for 25 min at 37°C. and the labeling stopped by addition of 0.9 ml ice-cold KRB-Hepes-BSA, 2.8 mM glucose, 1 mM (unlabeled) leucine and washed 3x in this buffer. The islet pellet was extracted by two cycles of freeze-thawing followed by sonication on ice in 200 $\mu$l 0.2 M glycine, 0.25% BSA, pH 8.8. The extracts were then centrifuged at 12,000 xg for 10 min to remove particulate material and the supernatants kept frozen at –20°C before analysis. Proinsulin and insulin were immunoprecipitated as described in detail previously [38]. In brief, duplicate samples of 50 $\mu$l aliquots of extracts from each islet preparation were incubated with 20 $\mu$l guinea-pig anti-insulin serum that cross-reacts with proinsulin (Sigma. MO, binding capacity more than 10-fold higher than total immunoreactive insulin in the 50 $\mu$l aliquot) and antibody-bound products then precipitated with protein-A Sepharose. Non-specific binding was measured in parallel using a single extract aliquot of 50 $\mu$l and without anti-insulin serum. Radioactivity was measured in a liquid scintillation counter (ReadyFlow III from Beckman Coulter, Fullerton, CA).

### 2.2.17 Statistical analysis

Samples were evaluated in a randomized manner by a single investigator (D. S.) blind to the treatment conditions. Data are presented as means $\pm$ SE and were analyzed by Student's t-test or by analysis of variance with a Bonferroni correction for multiple group comparisons.

## 2.3 Results

### 2.3.1 Fas regulates β-cell function

To assess the role of Fas on β-cell secretory function, we first used mice with a natural Fas mutation (lpr [39]) transferred onto a C57BL/6j inbred strain background. In contrast to the original MRL/MpJ$^{lpr}$ mice, that have an onset of a systemic lupus erythematosus-like autoimmune-disease at 8 weeks of age, the B6.MRL$^{lpr}$ mice do not develop any detectable sign of autoimmunity even at 13 weeks of age as described previously [40]. This was confirmed by the lack of detectable morphological and histological changes in the spleen, liver, kidney, lung and lymph nodes, as well as by the failure to detect circulating anti-nuclear antibodies or increased circulating cytokine (IL-1$\beta$, IFN$\gamma$, IL-6 and TNF$\alpha$) levels in Fas deficient mice between 7 to 13 weeks of age. At 8 weeks of age, Fas deficient B6.MRL$^{lpr}$ mice displayed impaired glucose tolerance after an intra peritoneal glucose load (Fig. 2.1 A & B). Normal sensitivity of the Fas deficient mice to injected insulin (Fig. 2.1 C), as well as unaltered insulin-stimulated glucose uptake in adipose tissue (Fig. 2.1 D), ruled out the possibility that insulin resistance was responsible for the impaired glucose tolerance. Accordingly, fasting (12h) circulating insulin ($0.59 \pm 0.15$ and $0.58 \pm 0.11$ ng/ml for wildtype and Fas deficient, respectively) and glucagon ($76 \pm 19$ and $77 \pm 15$ pg/ml) levels were unchanged. When measuring insulin secretion during the glucose tolerance test, baseline circulating insuin was higher in Fas deficient mice, but did not increase in response to the glucose load, whereas in wildtype mice there was a significant increase (Fig. 2.1 E). To exclude a non-specific effect related to the genetic background or an indirect effect due to Fas-deficiency in non-β-cells, a second set of glucose tolerance tests was performed on mice with a β-cell-specific knockout of Fas. Since these mice are on the NOD genetic background, care was taken to analyze the animals at 6 weeks of age, before the onset of insulitis. Compared to their wildtype NODβFas$^{+/+}$ littermates, heterozygous NODβFas$^{+/-}$ showed a slight, albeit non-significant impairment of glucose tolernace, whereas the homozygous NODβFas$^{-/-}$ mice displayed a significantly impaired glucose tolerance, thus confirming the selective effect of Fas deficiency (Fig. 2.1 F). Finally, the consequence of Fas deficiency was confirmed by a third animal model, i./e./ in mice with a β-cell-specific knockout of Fas on a C57Bl/6 genetic background (Fig. 2.1 G & H).

To characterize the defect in insulin secretion, we performed in situ pancreas perfusion experiments. In Fas deficient mice, the first phase insulin secretory response to a 15 min perfusion of 16.7 mM glucose was delayed and blunted, followed by an ablation of second phase insulin release (Fig. 2.1 I & J). The secretory defect apparent in the Fas deficient mouse pancreas was also present in isolated islets. Indeed, the ratio of high to low glucose-stimulated insulin release was 45% less than in wildtype islets (Fig. 2.1 K). The defect was also apparent in islets after 24 hours of culture (2-fold less glucose stimulated insulin secretion in the Fas deficient islets). The basal secretion was elevated in Fas deficient islets which is in agreement with the in vivo levels of insulin secretion and may reflect the compensatory increase in islet β-cell mass due to defective secretion. The role of Fas in glucose-stimulated

insulin secretion was also verified in human islets by means of the antagonistic anti-Fas antibody ZB4. In cultured human islets isolated from pancreata of organ donors, ZB4 inhibited glucose-stimulated insulin release already after 6 hours (Fig. 2.1 L), whereas it was enhanced by FasL after 48 hours (Fig. 2.1 M), supporting the notion that Fas activation is necessary for normal β-cell function.

Immunohistochemical evaluation of pancreata from 8 week old Fas deficient mice revealed a normal islet structure and ratio of β to $\alpha$-cells. However, islet β-cell mass was dramatically increased as compared to wildtype mice ($2.10 \pm 0.48$ mg in Fas deficient vs. $0.59 \pm 0.15$ mg in wildtype mice, $p < 0.01$), with no change in pancreas weight ($0.27 \pm 0.02$ g in Fas deficient vs $0.22 \pm 0.01$ g in wildtype mice). Despite the approximately threefold increase in islet β-cell mass of Fas deficient mice, insulin content per pancreatic wet weight and per isolated islet, remained unchanged (not shown), probably due to a severe decrease in insulin mRNA per β-cell (Fig. 2.2 A). In parallel, expression of PDX-1 was decreased at mRNA and protein level (Fig 2.2 A & B) whereas uncoupling protein 2 (used as a control) was not significantly changed (Fig. 2.2 A). Similarly, in cultured human islets, the antagonistic anti-Fas antibody ZB4 inhibited insulin and PDX-1 mRNA expression, demonstrating a direct regulation of these genes by Fas (Fig. 2.2 C). Furthermore, expression levels of FLIP mRNA were similar in Fas deficient and wildtype mice (not shown).

To characterize the secretory pathway regulated by Fas signaling, insulin release was stimulated with metabolic secretagogues, generating mitochondrial coupling factors such as ATP. The energy substrates, leucine as well as the cell permeable dimethyl esters of $\alpha$-ketoglutarate and succinic acid, were less effective in stimulating insulin release from Fas deficient mice and ZB4-treated human islets (Fig. 2.2 D & E). In contrast, the secretory responses in Fas deficient or ZB4-treated islets were restored in the presence of the phosphodiesterase inhibitors forskolin and 3-isobutyl-1-methylxanthine (IBMX) that increase cAMP levels (Fig. 2.2 D & E). Furthermore, acute glucose stimulated insulin synthesis (that reflects translation) was normal (Fig. 2.2 F). This suggests that the exocytotic machinery and insulin translation are fully functional in the absence of Fas-signaling, and indicates a defect in the stimulus-secretion coupling in addition to persistently decreased insulin mRNA expression and insulin content. Consistent with this hypothesis, ATP concentration was decreased in Fas deficient and ZB4-treated islets (Fig. 2.2 G & H).

### 2.3.2 Fas and FasL are expressed in islets and regulate insulin and PDX-1 mRNA expression

FasL and, at low levels, the Fas receptor are normally expressed in β-cells, as previously shown in human islets and in the present study in mouse islets [[7, 10] and Fig. 2.3 A–D]. To examine whether the effects of the Fas pathway indeed influence the phenotype of the β-cell, wildtype and Fas deficient mouse islets were transfected with a vector encoding Fas (Fig. 2.3 C & D). Transfection efficiency in β-cell monolayers was approximately 30–50% (Fig. 2.3 D). Restoration of Fas in Fas deficient islets significantly stimulated insulin and PDX-1 mRNA expression whereas overexpression of Fas at similar levels in wildtype mouse islets did not affect these genes,

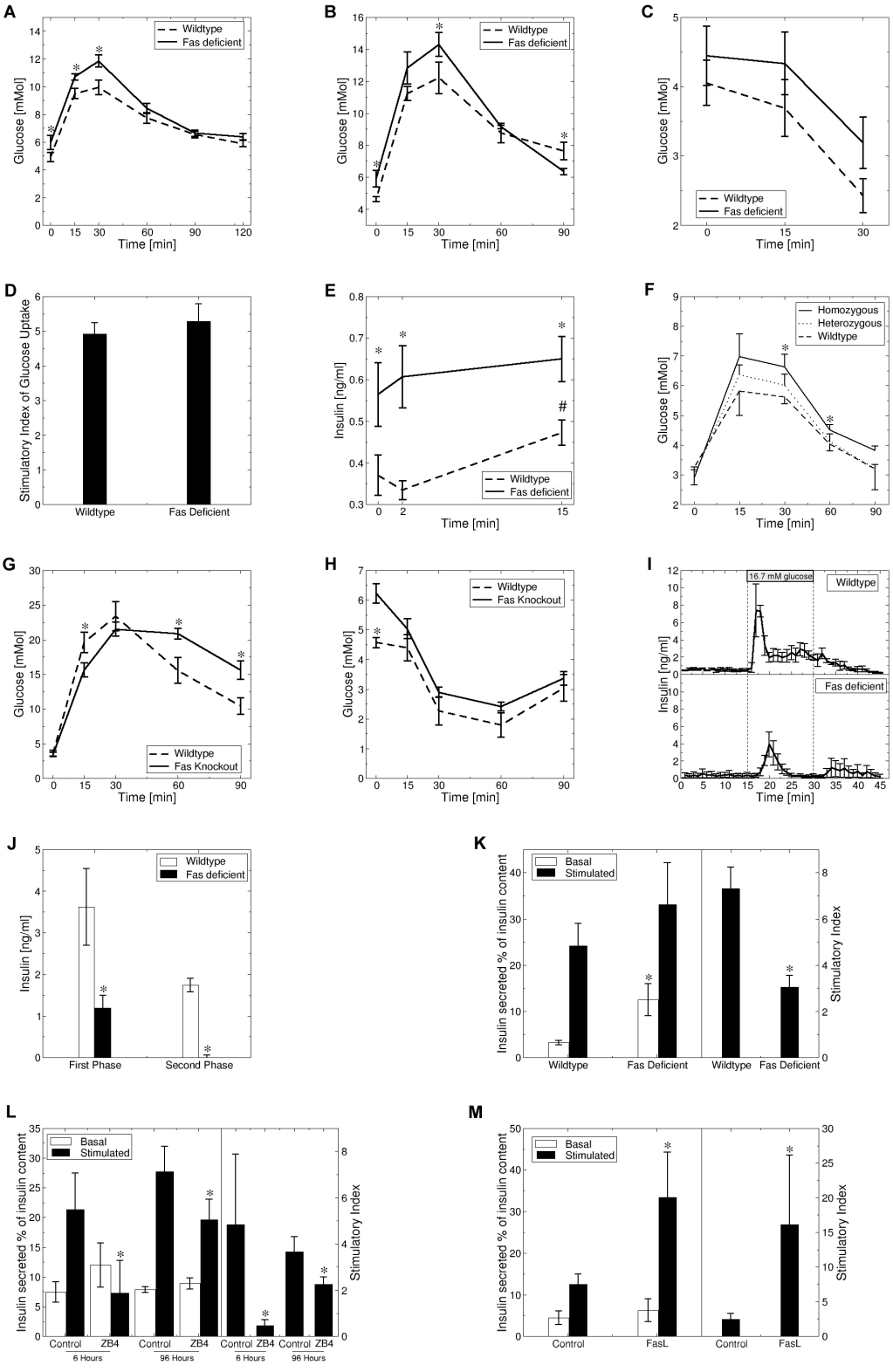
A
Wildtype
Fas deficient
Glucose [mMol]
Time [min]
B
Wildtype
Fas deficient
Glucose [mMol]
Time [min]
C
Wildtype
Fas deficient
Glucose [mMol]
Time [min]
D
Stimulatory Index of Glucose Uptake
Wildtype
Fas Deficient
E
Insulin [ng/ml]
Wildtype
Fas deficient
Time [min]
F
Homozygous
Heterozygous
Wildtype
Glucose [mMol]
Time [min]
G
Glucose [mMol]
Wildtype
Fas Knockout
Time [min]
H
Wildtype
Fas Knockout
Glucose [mMol]
Time [min]
I
16.7 mM glucose
Wildtype
Fas deficient
Insulin [ng/ml]
Time [min]
J
Wildtype
Fas deficient
Insulin [ng/ml]
First Phase
Second Phase
K
Basal
Stimulated
Insulin secreted % of insulin content
Stimulatory Index
Wildtype
Fas Deficient
Wildtype
Fas Deficient
L
Basal
Stimulated
Insulin secreted % of insulin content
Stimulatory Index
Control
ZB4
Control
ZB4
Control
ZB4
Control
ZB4
6 Hours
96 Hours
6 Hours
96 Hours
M
Basal
Stimulated
Insulin secreted % of insulin content
Stimulatory Index
Control
FasL
Control
FasL

**Figure 2.1:** Fas regulates β-cell secretory function. Blood glucose levels following i.p. injection of **(A)** 2 mg glucose and **(B)** 1 mg glucose / g body weight in male Fas deficient B6.MRL$^{lpr}$ and wildtype C57BL/6j mice aged 7–8 weeks. * indicates $p < 0.05$, Fas deficient versus wildtype; $n = 15$ and 10 for each group in **(A)** and **(B)**, respectively. **(C)** Blood glucose levels following i. p. injection of insulin in male Fas deficient and wildtype mice. * indicates $p < 0.05$, Fas deficient vs. wildtype; $n = 15$ for each group. **(D)** Insulin-stimulated 2-Deoxyglucose uptake in adipocytes isolated from Fas deficient and wildtype mice. Data are the means of 2 separate experiments, each in hexaplicates. **(E)** Insulin levels following i.p. injection of 2 mg glucose / g body weight in male Fas deficient and wildtype mice; $n = 15$ for each group. * indicates $p < 0.05$ relative to wildtype and # indicates $p < 0.05$ relative to timepoint 0 and 2 min. **(F & G)** Blood glucose levels following i.p. injection of glucose in **(F)** male prediabetic NODβFas$^{-/-}$ (homozygous), NODβFas$^{+/-}$ (heterozygous), and NODβFas$^{+/+}$ (wildtype) littermate mice aged 5–6 weeks,* indicates $p < 0.05$, NODβFas$^{-/-}$ vs. NODβFas$^{+/+}$, $n = 7$, 5 and 10, respectively for each group and in **(G)** male BL6βFas$^{-/-}$ (Fas Knockout) and BL6βFas$^{+/+}$ (wildtype) littermate mice aged 9–10 weeks. * indicates $p < 0.05$, $n = 12$ and 5 for each group, respectively . **(H)** Blood glucose levels following i.p. injection of insulin in male Fas knockout and wildtype littermate mice. * indicates $p < 0.05$, $n = 16$ and 4 for each group, respectively. **(I & J)** Glucose induced insulin secretion in perfused pancreata from Fas deficient and wildtype mice. Pancreata were perfused with basal solution (2.8 mM glucose) for 30 min before perfusate was collected (from time 0). Glucose was increased to 16.7 mM glucose for the indicated period. First phase insulin secretion for wildtype mice was calculated from min 16 to 20, and for Fas deficient mice from min 17 to 25. Second phase insulin secretion for wildtype mice was calculated from min 20 to 30 and for Fas deficient mice from min 25 to 30. Data is the mean of 3 separate animals for each group. **(K)** Percentage of islet insulin content released during successive 1 h incubation at (basal) 3.3 mM and (stimulated) 16.7 mM glucose following an 8 day culture period of islets isolated from Fas deficient and wildtype mice, and (right panel) the corresponding stimulatory index of insulin secretion. Data are the means of 4 separate experiments, each in hexaplicate. Insulin content for wildtype mice was $19.77 \pm 2.71$ pMol and for Fas deficient mice, $24.75 \pm 4.50$ pMol. * indicates $p < 0.001$. **(L)** Percentage of islet insulin content released during successive 1 h incubation at (basal) 3.3 and (stimulated) 16.7 mM glucose following a 6 hour and 4 day incubation period of islets isolated from human pancreata in the presence of 500 ng/ml isotype IgG (control) or 500 ng/ml antagonistic anti-Fas antibody (ZB4), and (right panel) the corresponding stimulatory index of insulin secretion. Data are the means of 3 separate experiments, each in triplicate. Insulin content for control islets was $6.12 \pm 2.41$ and $4.50 \pm 0.26$ pMol and for ZB4-treated islets, $7.92 \pm 1.50$ and $4.43 \pm 0.79$ pMol after 6 hours and 4 days. * indicates $p < 0.001$. **(M)** Percentage of islet insulin content released during successive 1 h incubations at basal and stimulated glucose following incubation of human islets in the presence of FasL for 48 hours. Insulin content for control was $6.12 \pm 2.41$ and for FasL-treated islets $9.65 \pm 2.02$ pMol. Data are the means of 2 separate experiments, each done in quintaplicate.

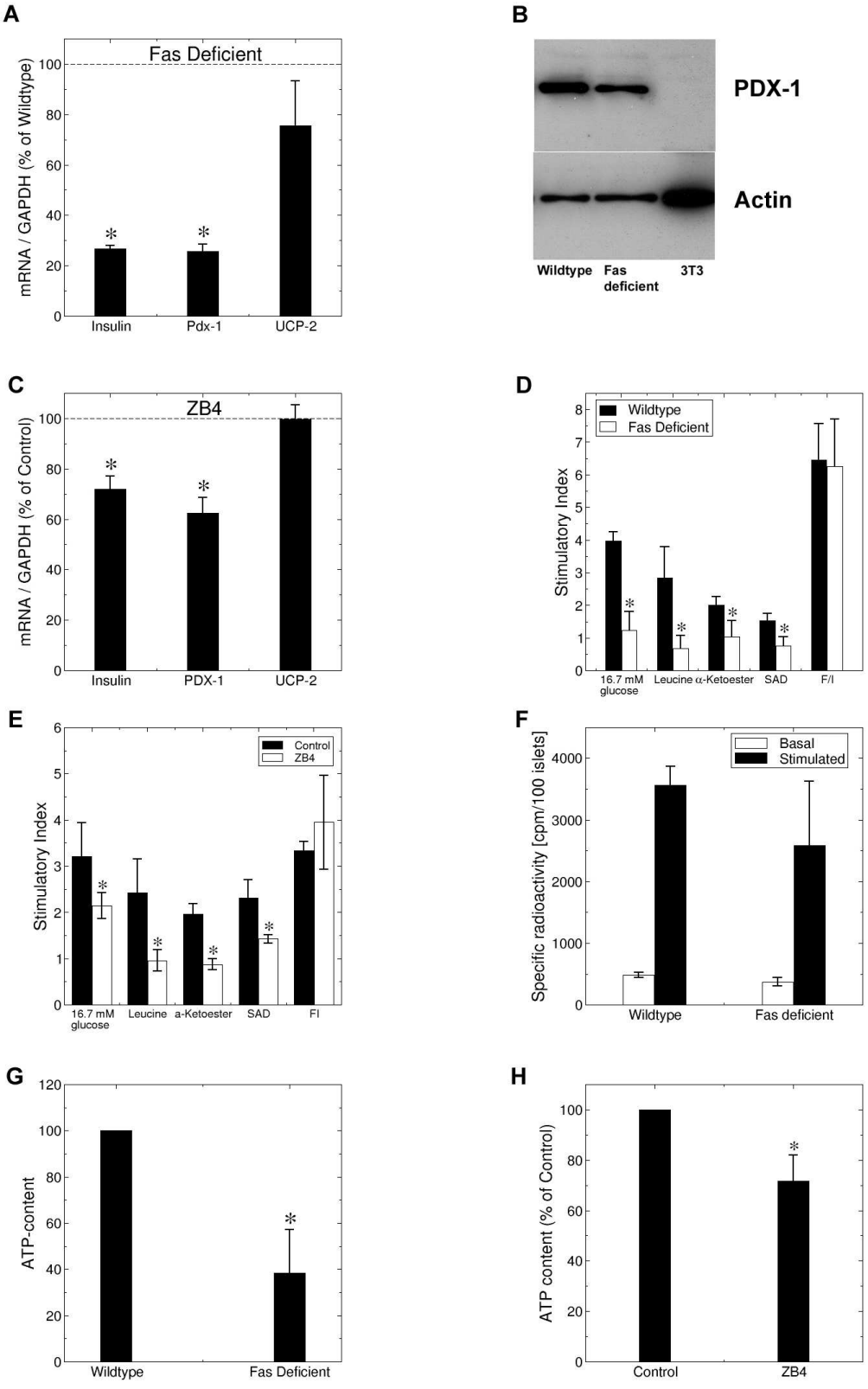
A
Fas Deficient
mRNA / GAPDH (% of Wildtype)
Insulin
Pdx-1
UCP-2
B
PDX-1
Actin
Wildtype
Fas deficient
3T3
C
ZB4
mRNA / GAPDH (% of Control)
Insulin
PDX-1
UCP-2
D
Wildtype
Fas Deficient
Stimulatory Index
16.7 mM glucose
Leucine
α-Ketoester
SAD
F/I
E
Control
ZB4
Stimulatory Index
16.7 mM glucose
Leucine
a-Ketoester
SAD
FI
F
Basal
Stimulated
Specific radioactivity [cpm/100 islets]
Wildtype
Fas deficient
G
ATP-content
Wildtype
Fas Deficient
H
ATP content (% of Control)
Control
ZB4

**Figure 2.2:** **(A)** Quantitative RT-PCR detection of insulin, PDX-1 and uncoupling protein 2 (UCP-2) mRNA expression. Total RNA was isolated from Fas deficient and wildtype islets. The level of mRNA expression was normalized to GAPDH and the results expressed as percentage of wildtype islet mRNA levels; $n = 5$ for each group. * denotes $p < 0.05$. **(B)** Immunoblotting of PDX-1 and Actin. Protein was isolated from Fas deficient and wildtype islets, and from the 3T3 cell-line (negative control). One of two experiments is shown. **(C)**Quantitative RT-PCR detection of insulin, PDX-1 and uncoupling protein 2 (UCP-2) mRNA expression. Total RNA was isolated from human islets cultured for 4 days in the presence of 500 ng/ml isotype IgG (control) or 500 ng/ml antagonistic anti-Fas antibody (ZB4). The level of mRNA expression was normalized to GAPDH and the results expressed as percentage of control islet mRNA levels. Data are the means of 3 separate experiments, each in duplicate. **(D & E)** Stimulatory index of insulin secretion during successive 1 h incubation at (basal) 3.3 mM glucose and (stimulated) 16.7 mM glucose, 20 mM leucine, 10 mM $\alpha$-ketoglutarate dimethyl ester ($\alpha$-Ketoester), 10 mM succinic acid dimethyl ester (SAD), or 10 M forskolin and 100 M IBMX (F/I), **(D)** following a 4 day culture period of islets isolated from Fas deficient and wildtype mice and **(E)** after a 4 day culture period of human islets from 3 different donors in the presence of 500 ng/ml isotype IgG (control) or 500 ng/ml antagonistic anti-Fas antibody (ZB4). Data are the means of 3 separate experiments, each in triplicate. * indicates $p < 0.05$ vs. wildtype or controls. **(F)** Islets isolated from wildtype and Fas deficient mice were radiolabeled (25 min) wiht [3H]-Leucine in 2.8 mM (basal) and 16.7 mM (stimulated) glucose. Radiolabeled proinsulin plus insulin is presented as specifically immunoprecipitable radioactivity as a function of islet number (cpm/100 islets). Data represent the means of 3 separate animals per group. **(G)** After 1 day in culture, islets isolated from Fas deficient and wildtype mice were incubated successively for 30 min in 2.8 mM glucose followed by an additional 10 min in 16.7 mM glucose and analyzed for stimulated ATP content. * indicates $p < 0.001$. Data are means of percentage relative to control for 3 separate experiments, each in triplicate. **(H)** After a 4 day culture period, human islets cultured in the presence of 500 ng/ml isotype IgG (control) or 500 ng/ml antagonistic anti-Fas antibody (ZB4), were incubated successively for 30 min in 2.8 mM glucose followed by an additional 10 min in 16.7 mM glucose and analysed for stimulated ATP-content. * indicates $p < 0.05$. Data are means of percentage relative to control for islets from 3 different donors, each plated in triplicate.

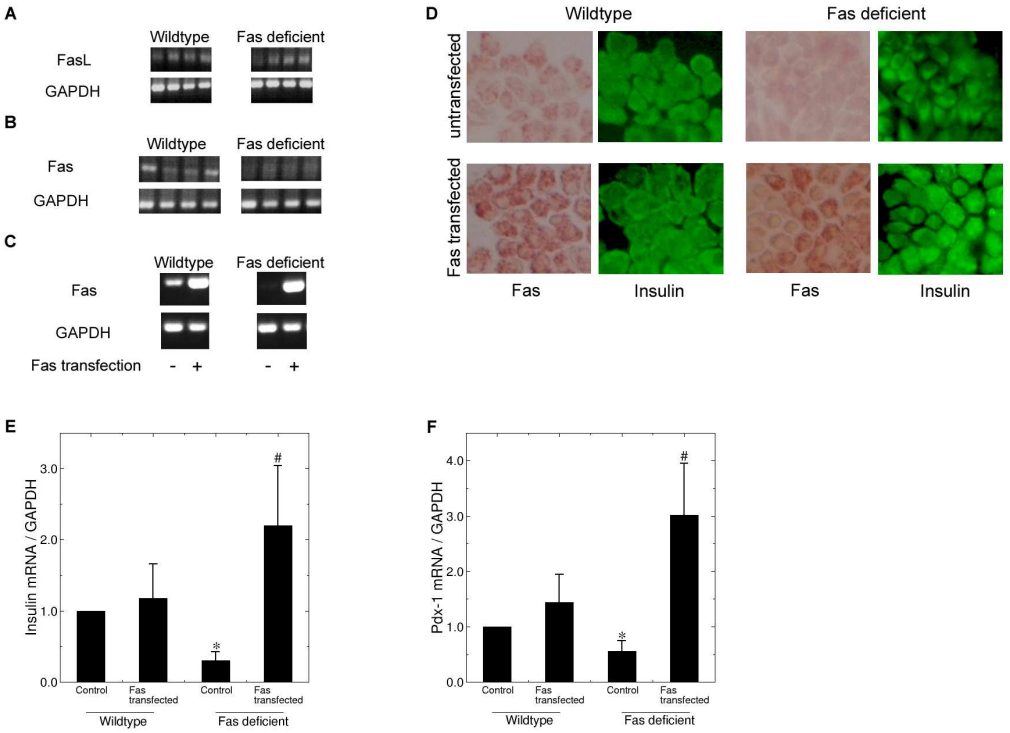


**Figure 2.3:** Fas and FasL are expressed in mouse islets and regulate insulin and PDX-1 mRNA expression. RT-PCR analysis of **(A)** FasL and **(B)** Fas expression in wildtype and Fas deficient islets. Each lane represents an individual animal. GAPDH was used as control. **(C–F)** Fas deficient and wildtype islets were transfected with a vector encoding for Fas and incubated for 8 days: **(C)** RT-PCR of Fas and GAPDH (control) expression in Fas deficient and wildtype islets; **(D)** Immunostaining for Fas; Quantitative RT-PCR detection of **(E)** insulin and **(F)** PDX-1 mRNA expression normalized to GAPDH. $n = 3$ for each group. * denotes $p < 0.05$ relative to wildtype and # relative to Fas deficient control.

possibly because endogenous Fas was sufficient to maximally stimulate steady-state PDX-1 levels (Fig. 2.3 E & F).

### 2.3.3 FLIP regulates insulin and PDX-1 mRNA expression via the alternative pathway of NF-$\kappa$B

Next we investigated the mediators of Fas regulating insulin production. Transfection efficiency of FLIP in β-cell monolayers was approximately 30–50%, as previously shown [14]. Overexpression of FLIP significantly increased islet insulin and PDX-1 mRNA and protein expression accompanied by increased insulin content (Fig. 2.4 A, B & C). Next we tested whether FLIP can also regulate insulin production in Fas deficient mouse islets. As expected, transfection of FLIP in Fas deficient islets enhanced islet insulin content by $63 \pm 24\%$ ($n = 3$, $p = 0.05$). The functional role of FLIP in β-cell maturation was then investigated by RNA interference (siRNA). siRNA to FLIP suppressed endogenous FLIP expression by 70% leading to a 50% decrease in insulin and PDX-1 expression with a concomitant de-

crease in insulin content (Fig. 2.4 D) whereas scrambled siRNA had no effect on these genes (not shown). Beta-cell proliferation and apoptosis remained unaffected by transfection with FLIP at baseline 11.1 mM glucose (not shown), in support of the concept that Fas-FLIP can affect β-cell function independently of changes in cell turnover. To test whether FLIP acts via NF-$\kappa$B, INS-1E cells were transfected with an NF-$\kappa$B-driven Luciferase construct. Co-transfection with FLIP strongly induced NF-$\kappa$B activity, reaching similar levels to IL-1β (Fig. 2.4 E). Nevertheless, detection of p50-RelA (p65) binding to an NF-$\kappa$B consensus site revealed no significant change following transfection with FLIP (Fig. 2.4 F). However, using an anti-RelB antibody which detects binding to an NF-$\kappa$B consensus site, revealed a robust induction of RelB by FLIP, indicating NF-$\kappa$B activation via the alternative pathway (Fig. 2.4 G). To support these results, we used islets isolated from aly/aly mice, which are defective in activation of the alternative NF-$\kappa$B pathway because of a mutation in NIK [41]. In contrast to wildtype islets, in aly/aly mice, FLIP failed to induce insulin and PDX-1 mRNA expression (Fig. 2.4 H & I). Finally, aly/aly mice displayed impaired glucose tolerance (Fig. 2.4 J), supporting the concept that NF-$\kappa$B activity is required for β-cell secretory function [16].

## 2.4 Discussion

Hitherto, activation of the Fas receptor, was thought to mainly activate the apoptotic cascade. Recently we have shown that in human β-cells FLIP may switch Fas-signaling towards proliferation [14]. Here we demonstrate that Fas is also a regulator of β-cell function. This is supported by impaired glucose tolerance in Fas deficient animals, and by an abnormal response to glucose in perfused pancreata and in isolated islets. In particular, impaired β-cell function in the perfused pancreas was characterized by a delayed and reduced first and second phase of insulin secretion. As shown in isolated islets, this defect is explained by decreased ATP synthesis, and suggests a mitochondrial defect. Indeed, decreased total cellular ATP mainly reflects overall mitochondrial dysfunction [42]. This notion is supported by the failure of energy substrates generating mitochondrial coupling factors to produce a normal insulin release. In contrast, non-fuel stimuli evoked normal insulin secretion, suggesting that metabolism-secretion coupling is defective in the absence of Fas-signaling while the exocytotic machinery is fully functional.

The defective metabolism-secretion coupling may be a consequence of decreased PDX-1 expression. PDX-1 has recently been shown to be an essential regulator of several events in insulin secretion, including mitochondrial metabolism and in particular ATP production [19, 21]. In line with these findings, we observed a decrease in PDX-1 expression in the islets of Fas deficient animals. Moreover, FLIP upregulation increased insulin and PDX-1 expression. Interestingly, mutations in PDX-1 have been associated with type 2 diabetes [43, 44] and high glucose dramatically lowers DNA-binding activity of PDX-1 [45].

Nonobese diabetic (NOD) mice develop spontaneous autoimmune diabetes, but Fas deficient NOD mice are protected against the disease [46–49]. Therefore, Fas has been postulated to play an important role in the β-cell demise in type 1 diabetes.

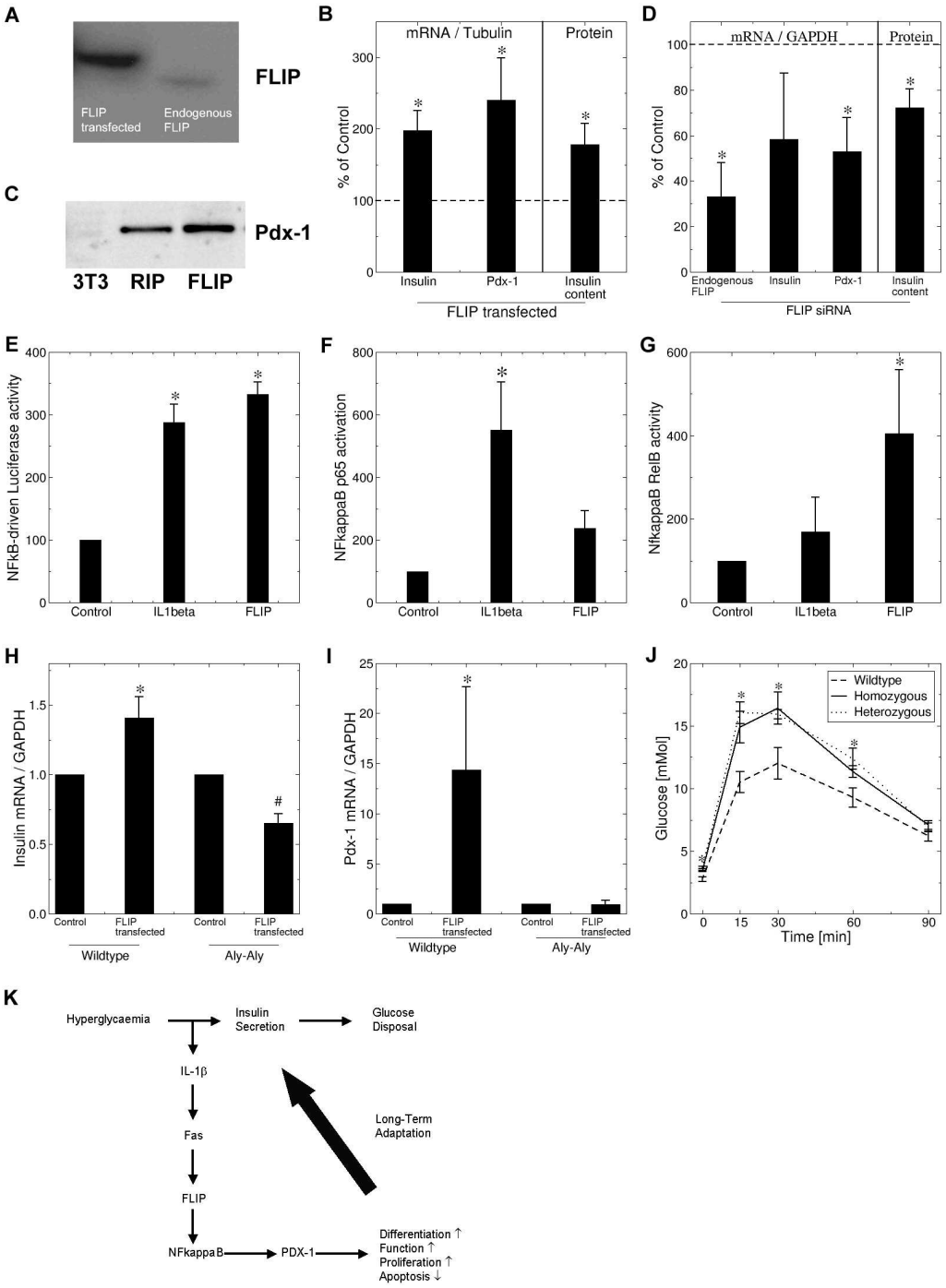
A
FLIP
FLIP transfected
Endogenous FLIP
B
mRNA / Tubulin
Protein
% of Control
Insulin
Pdx-1
Insulin content
FLIP transfected
C
Pdx-1
3T3
RIP
FLIP
D
mRNA / GAPDH
Protein
% of Control
Endogenous FLIP
Insulin
Pdx-1
Insulin content
FLIP siRNA
E
NFkB-driven Luciferase activity
Control
IL1beta
FLIP
F
NFkappaB p65 activation
Control
IL1beta
FLIP
G
NfkappaB RelB activity
Control
IL1beta
FLIP
H
Insulin mRNA / GAPDH
Control
FLIP transfected
Wildtype
Aly-Aly
I
Pdx-1 mRNA / GAPDH
Control
FLIP transfected
Wildtype
Aly-Aly
J
Wildtype
Homozygous
Heterozygous
Glucose [mMol]
Time [min]
K
Hyperglycaemia
Insulin Secretion
Glucose Disposal
IL-1β
Fas
FLIP
NFkappaB
PDX-1
Long-Term Adaptation
Differentiation ↑
Function ↑
Proliferation ↑
Apoptosis ↓

**Figure 2.4:** INS-1E **(A, E–G)**, wildtype and aly/aly mouse islet **(B–D,H,I)** cells were transfected with a mock or a vector encoding for FLIP (FLIP transfected), or exposed to siRNA to FLIP. **(A)** Immnunoblotting of FLIP. **(B, D)** Quantitative RT-PCR detection of insulin, PDX-1 and FLIP mRNA expression, and islet insulin content. The level of mRNA expression was normalized to tubulin or GAPDH and the results expressed as mRNA levels relative to controls. **(C)** Immunoblotting of PDX-1 in islets and in 3T3 cells (negative control) transfected with the RIP vector alone an dwith RIP-FLIP. One of two experiments is shown. Analysis of NF-$\kappa$B activity by **(E)** detection of Luciferase activity following transfection of an NF-$\kappa$B-driven Firefly Luciferase construct normalized to a co-transfected constitutive Renilla Luciferase construct, and by detection of **(F)** p50-p65 and **(G)** RelB binding to an NF-$\kappa$B consensus site. The effect of FLIP was compared to 150 pg/ml IL-1β. Data is a mean of 4 separate experiments each in duplicate. Quantitative RT-PCR detection of **(H)** insulin and **(I)** PDX-1 mRNA expression normalized to GAPDH. $n = 3$–4 independent experiments. * denotes $p < 0.05$ relative to wildtype controls and # $p < 0.05$ relative to aly/aly controls. **(J)** Blood glucose levels following an i. p. injection of glucose (2 mg/g body weight) in male homozygous and heterozygous aly/aly mice and C57Bl/6 (wildtype) mice. * indicates $p < 0.05$ homozygous, heterozygous vs wildtype, $n = 6$, 5 and 5 respectively for each group. **(K)** Hypothetical model illustrating the consequence of hyperglycaemia on β-cell production of IL-1β in parallel wiht insulin secretion. The paracrine effect of IL-1β induces Fas engagement, which in the presence of FLIP leads to β-cell proliferation, differentiation and increased function.

However, a direct role of Fas in β-cell destruction has been challenged [50, 51]. Possibly, some of these discrepancies can be explained by the physiological role of Fas. Indeed, deletion of Fas will protect from cytokine-induced cell death but it will also impair the normal insulin secretory function as shown in the present study.

The endocrine pancreas has a remarkable capacity to adapt to conditions of increased insulin demand, such as in obesity and pregnancy, by increasing its functional β-cell mass [1]. Taken together with previous findings we propose a hypothesis for long-term β-cell plasticity, attributing a central role to the Fas pathway (Fig. 2.4 K). According to this hypothesis, long-term adaptation of the β-cells to conditions of increased demand may be triggered by hyperglycaemic excursions [52]. These excursions elicit β-cell production of IL-1β [8] followed by Fas upregulation [7, 9]. At low concentrations of IL-1β and in the presence of FLIP, Fas engagement leads to β-cell proliferation [53] and enhanced function via NF$\kappa$B and PDX-1, as shown in the present study.

The contribution of insufficient insulin secretion to the development of type 2 diabetes is now widely accepted [1–3]. However, opinions diverge regarding the relative contribution of a decrease in β-cell mass versus an intrinsic defect in the secretory machinery of the β-cell. Here we demonstrate that the Fas pathway regulates β-cell secretory function in addition to its known role in β-cell-turnover. It follows that the adaptive mechanisms of function and mass share common regulatory pathways and will therefore act in concert. However, β-cell adaptation is not solely dependent upon the Fas pathway since Fas deficient animals exhibited a compensatory increase in β-cell mass. Nevertheless, the results support a novel role for the Fas pathway in regulating β-cell secretory function.

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# 3 Low Concentration of IL-1β induces FLIP-mediated β-cell proliferation in human pancreatic islets

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**Abstract**

High glucose concentrations have a dual effect on β-cell turnover, inducing proliferation in the short-term and apoptosis in the long-term. Hyperglycemia leads to β-cell production of IL-1β in human pancreatic islets. Fas, a death receptor regulated by IL-1β, is involved in glucose-induced β-cell apoptosis. Fas engagement can be switched from a death signal to induction of proliferation when the caspase-8 inhibitor FLIP is active. Here we show that IL-1β at low concentrations may participate in the mitogenic actions of glucose through the Fas-FLIP pathway. Thus, exposure of human islets to low IL-1β concentrations (0.01–0.02 ng/ml) stimulated proliferation and decreased apoptosis whereas increasing amounts of IL-1β (2–5 ng/ml) had the reverse effects. A similarly bimodal induction of FLIP, PDX-1 and Pax4 mRNA expression as well as glucose-stimulated insulin secretion was observed. In contrast, Fas induction by IL-1β was monophasic. Low IL-1β also induced the IL-1 receptor antagonist (IL-1Ra), suppression of which by RNA interference abrogated the beneficial effects of low IL-1β. The Fas antagonistic antibody ZB4 and siRNA to FLIP prevented low IL-1β-stimulated β-cell proliferation. Consistent with our in vitro results, IL-1β knockout mice displayed glucose intolerance along with a decrease in islet Fas, FLIP, Pax4 and PDX-1 transcripts. These findings indicate that low IL-1β levels positively influence β-cell function and turnover through the Fas-FLIP-pathway, and that IL-1Ra production prevents harmful effects of high IL-1β concentrations.

## 3.1 Introduction

The capacity of the pancreatic β-cell to adaptively increase insulin secretion in response to long term insulin resistance (for example in obesity) is fundamental to the maintenance of normoglycemia; failure of such a response results in diabetes

[1–8]. Changes in glucose concentration are key regulators of β-cell secretory function. Short-term exposure of human islets to increased glucose concentrations will enhance insulin production and β-cell proliferation, while prolonged exposure will have toxic effects leading to impaired insulin secretion and β-cell apoptosis [9–17]. Various mechanisms for this glucotoxic effect have been proposed, including advanced glycation end products, reactive oxygen species, impairment of insulin gene transcription and ER-stress. Recently, IL-1β was shown to be induced in β-cells of patients with type 2 diabetes indicating that the cytokine could be an important mediator contributing to glucotoxicity [18]. Consistent with the latter, β-cell expression of IL-1β correlates with appearance of hyperglycemia in several animal models of diabetes including the Psammomys obesus, the OLEFT rat, the GK rat and the human islet amyloid polypeptide transgenic rat [18–21, and Peter C. Butler, personal communication]. IL-1β has been shown to impair insulin release and to induce Fas expression enabling Fas-triggered apoptosis in rodent and human islets [16, 22–31]. Accordingly, increased glucose concentrations also induce β-cell expression of Fas in vitro and in vivo which will then be activated by the endogenous Fas ligand (FasL) [16, 28, 32, 33].

Interestingly, the beneficial short-term effects of high glucose on β-cell function and proliferation are also partly mediated by Fas. Indeed, in the presence of the caspase-8 inhibitor FLIP, Fas signalling switches from apoptosis to cell replication [34]. Furthermore, recently we demonstrated an additional role for the Fas pathway in regulating insulin production and release [35]. Additionally, low concentrations of IL-1β stimulate insulin release in rat islets [36]. Since glucose induces IL-1β, we hypothesized that IL-1β may also mediate beneficial effects of glucose. We show that low concentrations of IL-1β induce β-cell proliferation and enhance β-cell secretory function via the Fas-FLIP pathway, an effect facilitated by the concomitant IL-1Ra production.

## 3.2 Research design and methods

### 3.2.1 Animals

Ethical approval for mouse studies was granted by the Zürich Cantonal Animal Experimentation Committee. C57BL/6j wildtype (WT) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). IL-1β knockout (ILKO mice, on a C57BL/6j background, were produced by gene targeting as described previously [37]. Animals were housed at 22°C with a 12 h light-dark cycle (lights on at 07:00) and allowed free access to water and chow.

### 3.2.2 Intra-peritoneal glucose and insulin tolerance tests

Mice were fasted 12 h overnight and injected intra-peritoneally with 2 mg/g body weight glucose (40% glucose solution, Laboratorium Dr. G. Bichsel AG, Interlaken, Switzerland) or with 0.75 mU/g recombinant human (rh) insulin (Novo Nordisk A/S, Bagsværd, Denmark) for the glucose or insulin tolerance test, respectively.

Blood samples were obtained from tail-tip bleedings, and blood glucose concentration was measured with a Glucometer (Freestyle, Disetronic Medical Systems, Burgdorf, Switzerland).

### 3.2.3 Islet isolation and culture

Human islets were isolated from pancreata of nine organ donors at the University of Geneva Medical Center and at the University of Illinois at Chicago. Mouse islets were isolated as previously described [38]. The islets were cultured on extracellular matrix-coated plates derived from bovine corneal endothelial cells (Novamed Ltd., Jerusalem, Israel), allowing the cells to attach to the dishes and spread. Human islets were cultured in CMRL 1066 medium containing 5.5 mM glucose and mouse islets in RPMI 1640 medium containing 11.1 mM glucose, both supplemented with 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 10% FCS (Invitrogen Ltd., Carlsbad, CA), hereafter referred to as culture medium. Two days after plating, when most islets were attached and began to flatten, the medium was changed to culture medium containing 5.5, 11.1 or 33.3 mM glucose with or without 0.01–5 ng/ml rh IL-1β, 500 ng/ml rhIL-1Ra (R&D Systems Inc., Minneapolis, MN), 500 ng/ml antagonistic Fas antibody (ZB4; MBL, Nogoya, Japan) or transfected as described below.

### 3.2.4 RNA interference

RNAs of 21 nucleotides, designed to target human IL-1Ra (5′AUCUGCAGAGGCCUCCGCAtt3′/5′UGCGGAGGCCUCUGCAGAUtt3′), human $FLIP_L$ (Silencer Pre-designed siRNA) and scrambled siRNA were synthesized by Ambion (Austin, TE). siRNA was transfected using SiPortAmineTM, and transfection efficiency estimated with cy3 labeled siRNA using SilenceTM siRNA Labeling Kit (Ambion), as described previously [39].

### 3.2.5 Beta-cell replication and apoptosis

For β-cell proliferation studies, a monoclonal antibody against the human (Zymed Laboratories Inc., San Francisco, CA) or mouse Ki-67 antigen (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used. The free 3′-OH strand breaks resulting from DNA degradation were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique according to the manufacturer's instructions (In Situ Cell Death Detection Kit, AP; Boehringer, Mannheim, Germany) and as described previously in detail [14, 18]. Thereafter, islets were incubated for 30 minutes at 37°C with a guinea pig anti-insulin antibody (Dako, Carpinteria, CA), followed by detection using the streptavidin-biotin-peroxidase complex (Zymed) or a fluorescein-conjugated rabbit anti-guinea pig antibody (Dako).

### 3.2.6 Histochemical analysis

The pancreata were weighed, fixed in formalin and embedded in paraffin. Ten representative sections from each pancreas (spanning the width of the pancreas) were used in the analysis of β-cell mass. Tissue sections were deparaffinized, rehydrated and incubated with guinea pig anti-insulin antibody (Dako) followed by detection with a fluorescein-conjugated rabbit anti-guinea pig antibody (Dako). Subsequently, the specimens were labeled for glucagon with mouse anti-glucagon antibody (Dako), followed by detection with donkey anti-mouse Cy3-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Beta cell mass was analyzed using OpenlabTM software. The relative area of β-cells (green fluorescence) was determined by quantification of the cross-sectional β-cell area divided by the cross-sectional area of total tissue. The β-cell mass per pancreas was estimated as the product of the relative cross-sectional area of β-cells per total tissue and the weight of the pancreas. For detection of FLIP expression in β-cells, islets were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, incubated with rabbit anti-FLIP (R&D) followed by detection with donkey anti-rabbit Cy3-conjugated antibody (Jackson). Subsequently the specimens were stained for insulin as described above.

### 3.2.7 Western blot analysis

Equivalent amounts of protein from each treatment group were run on 15% SDS polyacrylamide gels. Proteins were electrically transferred to nitrocellulose filters and incubated with rabbit anti Caspase 8 (Stressgen, Victoria, BC, Canada), rabbit anti-human $FLIP_L$ (R&D), rabbit anti PDX-1 (kindly provided by Stefan Zahn, NovoNordisk, Bagsvrd, Denmark) or rabbit anti-actin (Cell signaling, Beverly, MA) antibodies followed by incubation with anti-rabbit IgG peroxidase-conjugated antibodies (Santa Cruz Biotechnology Inc.). Immune complexes were detected by chemiluminescence using LumiGLO (Cell Signalling).

### 3.2.8 RNA extraction and quantitative reverse transcription-PCR (RT-PCR)

Total RNA was extracted from the cultured islets by using the Rneasy mini kit (Qiagen, Basel, Switzerland) and RT-PCR was performed by using the SuperScript Double-Stranded cDNA synthesis kit according to the manufacturer's instructions (Life Technologies, Gibco, Gaithersburg, MD). For quantitative analysis, we used the Light Cycler quantitative PCR system (Roche, Basel, Switzerland) with a commercial kit (Light Cycler-DNA Master SYBR Green I; Roche). Mouse primers used were 5′TACGGGGTTTGTGAAAGGAG3′ and 5′CACATCATTCCCCAGGAAAC3′ (insulin); 5′GAGGACCCGTACAGCCTACA3′ and 5′CGTTGTCCCGCTACTACGTT3′ (PDX-1); 5′CTAAATTTGGTTGCCCCAGA3′ and 5′CTCCCATTATGGAGCCTGAA3′ ($FLIP_L$); 5′GCTCTTTTTGCCTGGGAGATC3′ and 5′CCCGAAGGACTCGATTGATAGA3′ (PAX4); 5′TATCAAGGAGGCCCATTTTG3′ and 5′GGTCAGGGTGCAGTTTGTTT (Fas); 5′GTGGCAGTGATGGCATGGAC3′ and

5′CAGCACCAGTGGATGCAGGG3′ (GAPDH). Human primers used were 5′CCACCTTGGGACCTGTTTAG3′ and 5′TGATGCCAGAGGAAGAGGAG3′ (PDX-1); 5′CCACCGGAATCGGACTATCTT3′ and 5′TACTGCCCACGCTGGAACTC3′ (PAX4); 5′GAGCAAGCCCCTAGGAATCT3′ and 5′GCCCTGAGTGAGTCTGATCC3′ ($FLIP_L$); 5′CTACCTAGTGTGCGGGGAAC3′ and 5′GCTGGTAGAGGGAGCAGATG3′ (insulin); 5′GCATCTGGACCCTCCTACCT3′ and 5′CAGTCTGGTTCATCCCCATT3′ (Fas); 5′AGAGTCGCGCTGTAAGAAGC3′ and 5′TGGTCTTGTCACTTGGCATC3′ (Tubulin); 5′AACAGCGACACCCACTCCTC3′ and 5′GGAGGGGAGATTCAGTGTGGT3′ (GAPDH), 5′TACGGGTCCTGGCATCTTGT3′ and 5′CCATTTGTGTTGGGTCCAGC3′ (Cyclophilin).

### 3.2.9 Insulin and IL-1Ra release, and insulin content

For acute insulin release in response to glucose, islets were washed and pre-incubated (30 min) in Kreb's Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose and 0.5% BSA. KRB was then replaced by KRB 2.8 mM glucose for 1 h (basal), followed by an additional 1 h in KRB 16.7 mM glucose. Islets were extracted with 0.18 N HCl in 70% ethanol for determination of insulin content. To determine total insulin content of the pancreas, the tissue was homogenized in 1 ml 0.18 N HCl in 70% ethanol and left overnight at 4°C. Insulin was determined using a human insulin RIA kit (CIS bio international, Gif-Sur-Yvette, France), which has similar affinity for both mouse and human insulin. Serum insulin was measured using Luminex technology according to the manufacturer's instructions (Linco Research Inc. St. Charles, MO). IL-1Ra release in the islet supernatant was measured by using human anti-IL-1Ra ELISA kits (R&D).

### 3.2.10 Statistical analysis

Samples were evaluated in a randomized manner by a single investigator (K. M.) who was blinded to the treatment conditions. Data are presented as means $\pm$ SE and were analyzed by Student's t-test or by analysis of variance with a Bonferroni correction for multiple group comparisons.

## 3.3 Results

#### Low concentrations of IL-1β induce β-cell proliferation, are anti-apoptotic and enhance β-cell secretory function.

Human islets were cultured on extracellular matrix-coated plates in the presence of increasing IL-1β concentrations for 4 days. Exposure to 0.02 ng/ml IL-1β induced a 2-fold increase in β-cell proliferation compared to controls, but exposure to higher concentrations of 2 and 5 ng/ml resulted in an approximately 1.5-fold decrease (Figure 3.1 A & B). By contrast, 0.01 ng/ml IL-1β reduced baseline β-cell apoptosis 2-fold whereas higher doses of 2 and 5 ng/ml IL-1β increased the apoptosis rate by 2.3- and 3.6-fold, respectively (Figure 3.1 A & C). These changes in β-cell turnover were

accompanied by a 1.6-fold increase in glucose stimulated insulin secretion (GSIS) at 0.02 ng/ml IL-1β and a 2.8-fold reduction by 2 ng/ml IL-1β (Figure 3.1 D & E). Insulin content of the islets was not significantly affected.

### Endo- and exogenous modulation of IL-1β signaling by IL-1Ra.

We have recently shown expression of IL-1Ra in human β-cells [39]. Interestingly secreted IL-1Ra will antagonise the effect of IL-1β. To highlight the potential cross talk between these two molecules, we repressed endogenous production of IL-1Ra by RNA interference. As previously shown, small interfering RNA directed to IL-1Ra (siIL-1Ra) with a transfection efficiency of 70% suppressed IL-1Ra mRNA by 69 $\pm$ 6% [39]. Blockade of endogenous IL-1Ra by siIL-1Ra did not significantly affect baseline β-cell proliferation (Figure 3.2 A) but led to a 2-fold increase in apoptosis, levels similar to the ones obtained with 2 ng/ml of IL-1β (Figure 3.2 B). Addition of 0.02 ng/ml IL-1β failed to increase β-cell proliferation and to further increase β-cell apoptosis (Figure 3.2 A & B). In contrast, addition of exogenous rhIL-1Ra (500 ng/ml) increased β-cell proliferation and protected from the deleterious effects of a high concentration, 2 ng/ml, of IL-1β. Furthermore, the compound protected cells against prolonged exposure to 33.3 mM glucose (Figure 3.2 A & B). Similarly, siIL-1Ra completely blocked GSIS in the absence or presence of 0.02 ng/ml IL-1β, whereas exogenous rhIL-1Ra prevented the impairment of GSIS in the presence of 2 ng/ml IL-1β or 33.3 mM glucose (Figure 3.2 C & D). Interestingly, the low concentration of 0.02 ng/ml IL-1β induced a 1.8-fold increase in the release of its receptor antagonist IL-1Ra into the culture medium (Figure 3.2 E).

### Low dose interleukin-1β-induced β-cell proliferation is mediated via the Fas-FLIP pathway and may involve PDX-1 and Pax4.

Next we investigated the underlying mechanisms of the proliferative effect of IL-1β. We hypothesized that the Fas pathway mediates IL-1β induced β-cell proliferation. To test this hypothesis, first we investigated whether low concentrations of IL-1β are capable of inducing Fas. Exposure of human islets to 0.02 ng/ml IL-1β for 4 days induced Fas expression to levels similar to those observed with higher concentrations of IL-1β and 33.3 mM glucose (Figure 3.3 A). However, low concentrations of IL-1β did not activate caspase-8, the most upstream caspase in the Fas apoptotic pathway, while higher concentrations than 0.02 ng/ml IL-1β caused cleavage of procaspase-8, releasing the active form of the protease (Figure 3.3 B). To examine whether the induction of proliferation by 0.02 ng/ml of IL-1β is caused by interaction of constitutively expressed FasL [16, 28] and up-regulated Fas, we used the Fas antagonistic antibody ZB4. ZB4 inhibited the proliferative effect of IL-1β (Figure 3.3C). Since Fas signaling may induce proliferation in the presence of FLIP [34], we analyzed FLIP protein modulation by increasing concentrations of IL-1β. Similar to IL-1β-mediated changes in β-cell proliferation (Figure 3.1 B), low concentrations of IL-1β induced FLIP expression, while higher concentrations were inhibitory (Figure 3.3 D & E). Of note, FLIP mRNA expression remained unchanged in agreement with previous studies showing regulation of FLIP only at protein level [34]. The

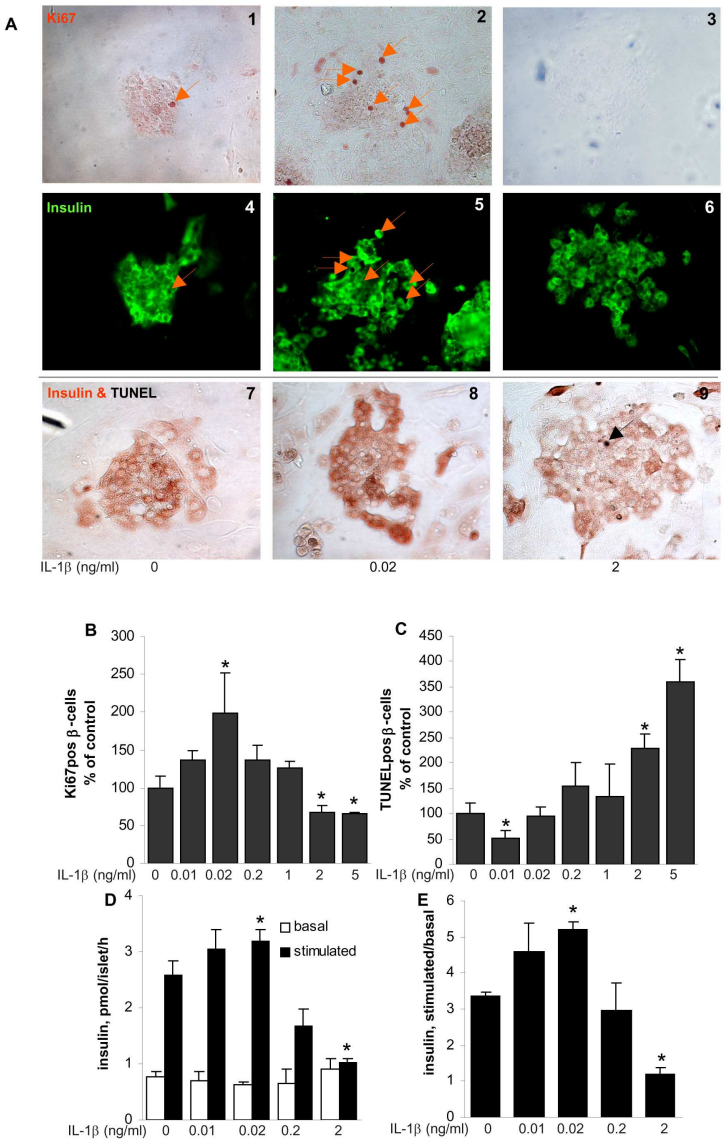



**Figure 3.1:** Low concentrations of IL-1β induce β-cell proliferation, are anti-apoptotic and enhance β-cell secretory function: Human islets were cultured on extracellular matrix-coated dishes for 4 days at 5.5 mM glucose with increasing concentrations of IL-1β. Double immuno-staining for β-cell proliferation with anti-Ki-67 in brown **(A 1–3)** and anti-insulin in green **(A 4–6)** and for β-cell apoptosis with the TUNEL assay in black and anti-insulin in brown **(A 7–9)**, in control islets **(A 1, 4, 7)** and in islets treated with 0.02 ng/ml **(A 2, 5, 8)** and with 2 ng/ml **(A 3, 6, 9)** IL-1β. The orange arrows mark β-cells stained positive for Ki-67 and insulin, and the black arrow marks a TUNEL positive β-cell. Percentage of Ki-67-positive **(B)** and TUNEL-positive **(C)** β-cells normalized to control incubations at 5.5 mM glucose alone (100%; in absolute values: $0.19 \pm 0.07\%$ Ki-67-positive β-cells and $0.40 \pm 0.05\%$ TUNEL-positive β-cells). The mean number of islets scored was 41 **(B)** and 52 **(C)** for each treatment condition from each donor. Basal and glucose-stimulated insulin secretion denote the amount secreted during successive 1-hour incubations at 2.8 mM (basal) and 16.7 mM (stimulated) glucose following the 4-day culture period expressed as secreted insulin **(D)** or stimulatory index **(E)**. Islets were isolated from four organ donors. Results are means ± SE. * $p < 0.05$ to untreated controls.

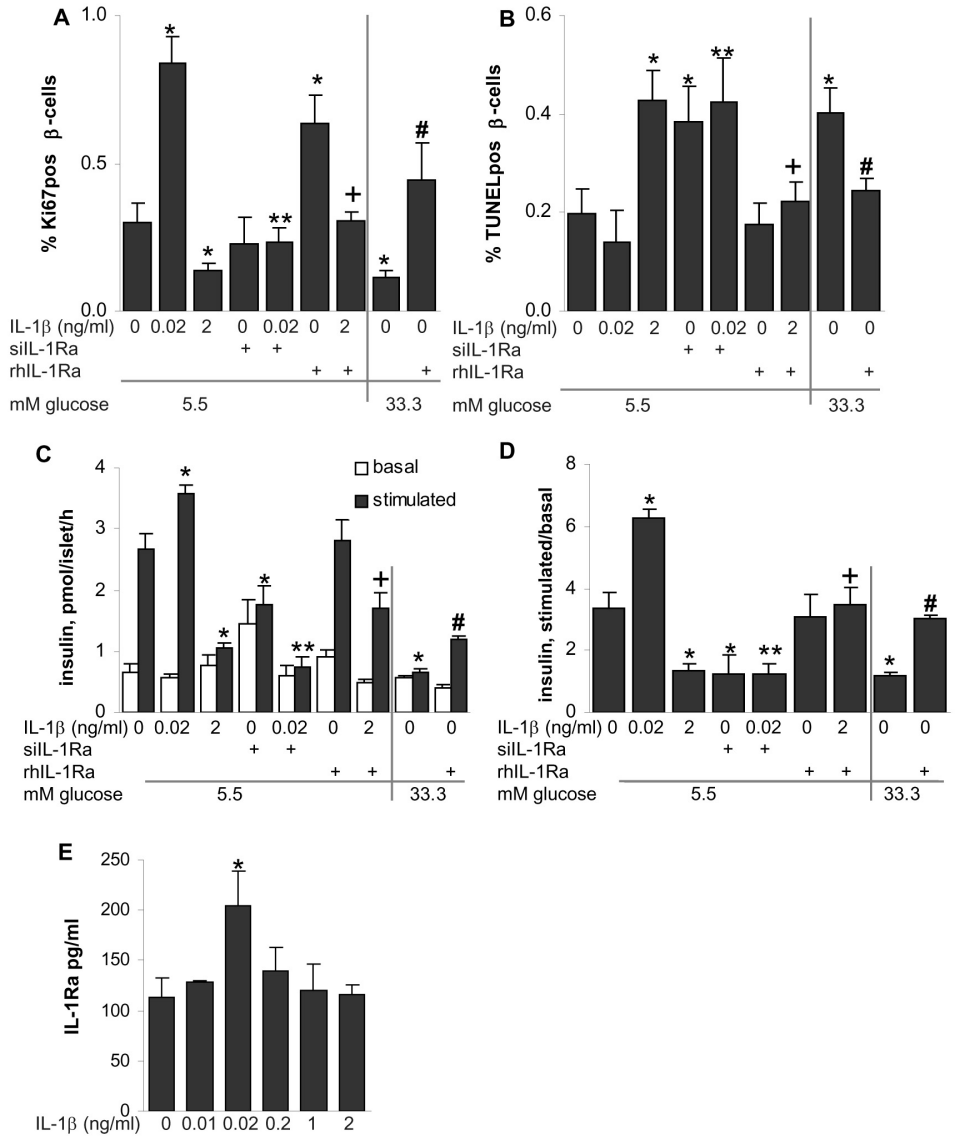



**Figure 3.2:** Endo- and exogenous modulation of IL-1β signaling by IL-1Ra: Human islets were cultured on extracellular matrix-coated dishes for 4 days at 5.5 or 33.3 mM glucose alone or with increasing concentrations of IL-1β or after transfection with 50 nM siIL-1Ra or with addition of 500 ng/ml exogenous recombinant human IL-1Ra. Results are means ± SE of percentage of Ki-67-positive **(A)** and TUNEL-positive **(B)** cells. The mean number of islets scored was 45 **(A)** and 43 **(B)** for each treatment condition from each donor. Basal and glucose-stimulated insulin secretion denote the amount secreted during successive 1-hour incubations at 2.8 mM (basal) and 16.7 mM (stimulated) glucose following the 4-day culture period expressed as secreted insulin **(C)** or stimulatory index **(D)**. **(E)** Secretion of IL-1Ra from human islets during 4 days of culture. Data were collected from three tubes per treatment in five separate experiments from five donors. Results are means ± SE. * $p < 0.05$ to untreated control. ** $p < 0.05$ to 0.02 ng/ml IL-1β alone. + $p < 0.05$ to 2 ng/ml IL-1β alone. # $p < 0.05$ to 33 mM glucose alone.

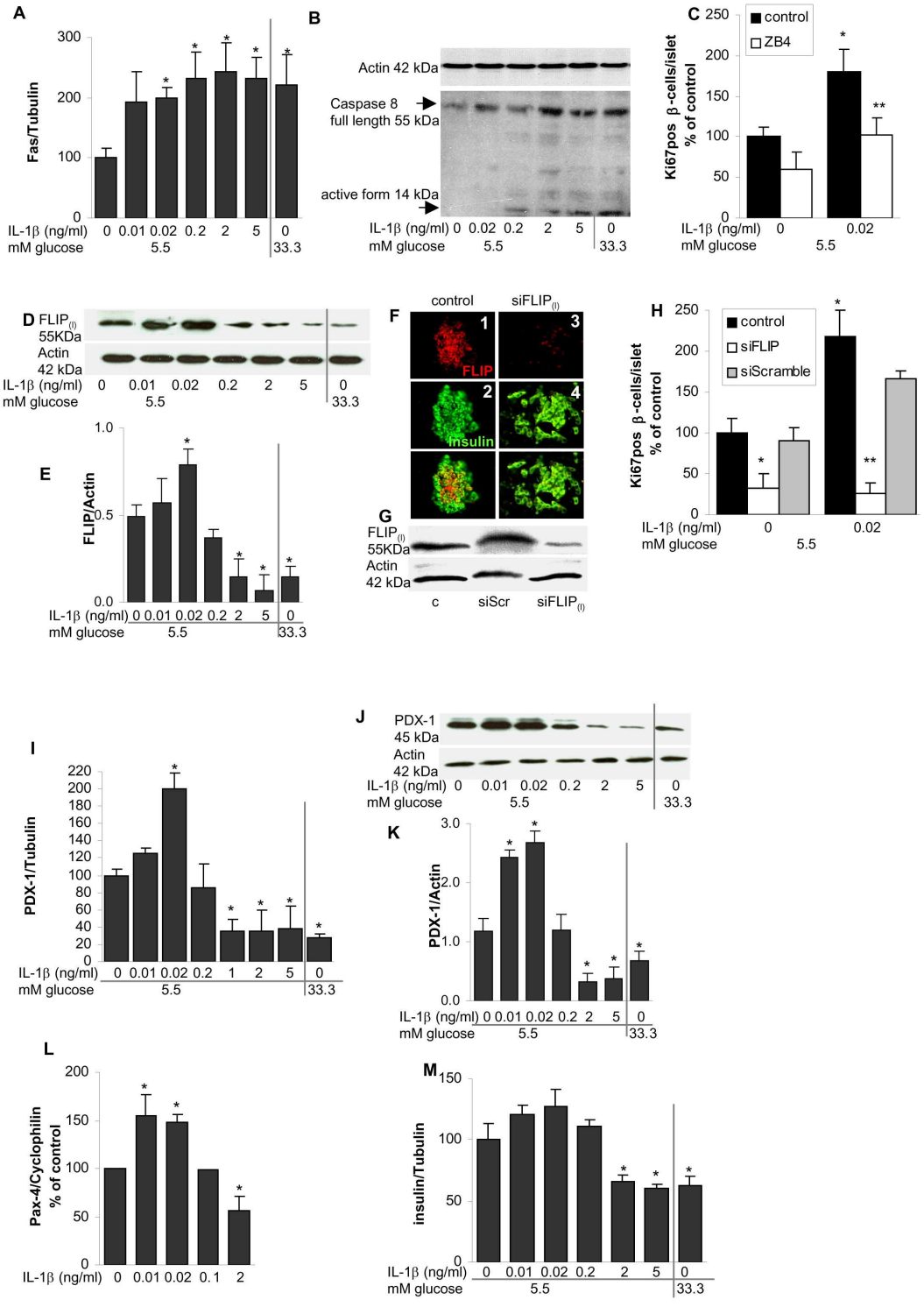
functional role of FLIP in IL-1β-induced β-cell proliferation was then investigated by RNA interference (siFLIP). siFLIP suppressed FLIP expression in most β-cells (Figure 3.3 F) leading to a 5.2-fold decrease in FLIP protein expression (for representative blot, see Figure 3G). Repression of FLIP resulted in impaired baseline β-cell proliferation and prevented IL-1β mediated replication, whereas scrambled siRNA had no effect on the number of Ki-67 positive β-cells (Figure 3.3 H). We next investigated the potential implication of the transcription factors PDX-1 and Pax4, known to promote β-cell replication [40–45]. Low concentrations of IL-1β stimulated PDX-1 mRNA and protein expression which were decreased by higher concentrations of IL-1β (Figure 3.3 I–K). Similarly, low concentrations of IL-1β stimulated Pax4 and insulin mRNA expression, which was decreased by higher concentrations (Figure 3.3 L & M). However, the stimulation of insulin mRNA by low IL-1β failed to reach statistical significance. Furthermore, Pax4 was stimulated at 12 hours but no longer after 4 days.

### Impaired glucose tolerance in IL-1β knockout mice.

To substantiate the role of IL-1β in normal glucose homeostasis in vivo, glucose tolerance tests were performed on IL-1β knockout (ILKO) mice. As predicted, we found that ILKO mice displayed glucose intolerance (Figure 3.4 A). Normal sensitivity of the ILKO mice to injected insulin (Figure 3.4 B) ruled out the possibility that insulin resistance was responsible for the impaired glucose tolerance. Food intake and body weight were similar in ILKO and WT mice (not shown). Analysis of pancreatic tissue of ILKO mice revealed a normal structure of the islets with no significant decrease in β-cell mass (Figure 3.4 C & D). However, insulin mRNA expression was strongly decreased (Figure 3.4 E) although pancreatic insulin content showed no significant changes ($5.05 \pm 1.04$ versus $4.0 \pm 0.7$ mol/g insulin per pancreas in normal versus ILKO mice, respectively). Consistent with our in vitro experiments, Fas, FLIP, PDX-1 and Pax4 were decreased in ILKO mice (Figure 3.4 E). Islets of ILKO mice exhibited a strong decrease in baseline β-cell proliferation and GSIS compared to WT mice, while apoptosis and insulin content were not significantly different (Figure 3.5). The resistance of ILKO islets to β-cell glucotoxicity was then studied. Chronic exposure of wildtype mouse islets to 33.3 mM glucose for 4 days impaired β-cell proliferation, induced apoptosis, impaired GSIS and decreased insulin content (Figure 3.5). In contrast, islets of ILKO mice were not further altered by high glucose concentrations (Figure 3.5), supporting the concept of IL-1β mediated glucotoxicity [18]. Finally, we have confirmed that glucose regulates IL-1β secretion in mouse islets ($2.85 \pm 0.8$ and $5.68 \pm 1.6$ pg/ml of IL-1β in islets cultured for 4 days in 11.1 and 33.3 mM glucose, respectively, $n = 5$) as described in human islets [18].

## 3.4 Discussion

The role of IL-1β and other cytokines in the pathogenesis of type 1 diabetes is well established [25, 46]. More recently the concept emerged that cytokines may also me-


A
Fas/Tubulin
IL-1β (ng/ml) 0 0.01 0.02 0.2 2 5 0
mM glucose 5.5 33.3
B
Actin 42 kDa
Caspase 8
full length 55 kDa
active form 14 kDa
IL-1β (ng/ml) 0 0.02 0.2 2 5 0
mM glucose 5.5 33.3
C
control
ZB4
Ki67pos β-cells/islet
% of control
IL-1β (ng/ml) 0 0.02
mM glucose 5.5
D
FLIP(l) 55KDa
Actin 42 kDa
IL-1β (ng/ml) 0 0.01 0.02 0.2 2 5 0
mM glucose 5.5 33.3
E
FLIP/Actin
IL-1β (ng/ml) 0 0.01 0.02 0.2 2 5 0
mM glucose 5.5 33.3
F
control
siFLIP(l)
FLIP
insulin
G
FLIP(l) 55KDa
Actin 42 kDa
c siScr siFLIP(l)
H
control
siFLIP
siScramble
Ki67pos β-cells/islet
% of control
IL-1β (ng/ml) 0 0.02
mM glucose 5.5
I
PDX-1/Tubulin
IL-1β (ng/ml) 0 0.01 0.02 0.2 1 2 5 0
mM glucose 5.5 33.3
J
PDX-1 45 kDa
Actin 42 kDa
IL-1β (ng/ml) 0 0.01 0.02 0.2 2 5 0
mM glucose 5.5 33.3
K
PDX-1/Actin
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mM glucose 5.5 33.3
L
Pax-4/Cyclophilin
% of control
IL-1β (ng/ml) 0 0.01 0.02 0.1 2
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insulin/Tubulin
IL-1β (ng/ml) 0 0.01 0.02 0.2 2 5 0
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**Figure 3.3:** Low dose IL-1β-induced β-cell proliferation is mediated via the Fas-FLIP pathway and involves PDX-1 and Pax4: Human islets were cultured on extracellular matrix coated dishes and exposed for 4 days to increasing IL-1β concentrations or 33.3 mM glucose or 500 ng/ml of the antagonistic anti-Fas antibody ZB4 or 50 nM siRNA to $FLIP_L$ ($siFLIP_L$) or to scrambled siRNA (siScr). **(A)** Quantitative RT-PCR analysis of Fas expression. **(B)** Representative immunoblotting of caspase 8 and actin. For detection of caspase-8, an antibody recognizing full length (procaspase-8; 55 kDa), and the 14 kDa processed active form of caspase-8 was used. **(C)** β-cell proliferation determined by double staining with anti-Ki-67 and anti-insulin. **(D)** Representative immunobloting of $FLIP_L$ and actin. (E) The density of expression levels was quantified after scanning, and normalised to actin levels. **(F)** Double immunostaining of the islets with anti-$FLIP_L$ **(1,3)** and anti-insulin **(2,4)** antibodies in control islets **(1,2)** and $siFLIP_L$ treated islets **(3,4)**. **(G)** Representative immunobloting of $FLIP_L$ and actin. **(H)** β-cell proliferation determined by double staining with anti-Ki-67 and anti-insulin. **(I)** Quantitative RT-PCR analysis of PDX-1 expression. **(J)** Representative immunoblotting of PDX-1 and actin. **(K)** The density of expression levels were quantified after scanning and normalised to actin levels. **(L)** Quantitative RT-PCR analysis of Pax4 in islets exposed for 12 h to IL-1β and **(M)** of insulin in islets exposed for 4 days to IL-1β. Results are means ± SE. In the LightCycler quantitative PCR system, the levels of Pax4 and insulin expression were normalized against tubulin or cyclophylin and the results were expressed as mRNA levels relative to control incubations. The antibodies were blotted on the same membrane after stripping, actin was used as loading control. Islets were isolated from six organ donors. * $p < 0.05$ to untreated controls, ** $p < 0.05$ to 0.02 ng/ml IL-1β.

diate nutrient induced β-cell dysfunction during the development of type 2 diabetes [5, 6, 47, 48]. Intriguingly, some of these cytokines can be produced by β-cells, including IL-6, IL-1β, IL-1Ra, and PANDER [18, 19, 39, 49–53]. The fact that the non-immune β-cell synthesizes cytokines suggests their implication in islet physiology. Accordingly, we observed beneficial effects of low concentrations of IL-1β on β-cell proliferation, apoptosis and secretory function.

A low concentration of IL-1β stimulated IL-1Ra which in turn stimulated β-cell proliferation. Likewise, adenoviral expression of IL-1Ra increases β-cell replication in rat islets [54]. At first sight, these results suggest a signalling function for IL-1Ra. However, this is unlikely since IL-1Ra only has a single IL-1 receptor binding domain and is therefore unable to recruit the IL-1 receptor accessory protein, the second chain of the receptor complex, which is believed to be necessary for signalling [55]. Therefore the proliferative effects of exogenous IL-1Ra probably result from a restoration of a beneficial ratio of IL-1 to IL-1Ra, and not from direct effects of the latter. Corroborating this hypothesis, repression of endogenous IL-1Ra was deleterious, probably due to unbalanced actions of IL-1.

Fas expression on the surface of pancreatic β-cells contributes to cytokine-induced apoptosis [30, 56]. However, when FLIP is activated, Fas becomes mitogenic [34]. We propose that the Fas-FLIP pathway is modulated by IL-1β. This is supported by a simultaneous elevation of Fas and FLIP proteins at low IL-1β concentrations leading to β-cell proliferation. In contrast, higher concentrations of IL-1β decreased FLIP while Fas remained elevated leading to decreased proliferation and induction of apoptosis. Furthermore, the Fas antagonistic antibody ZB4 and siRNA to FLIP,

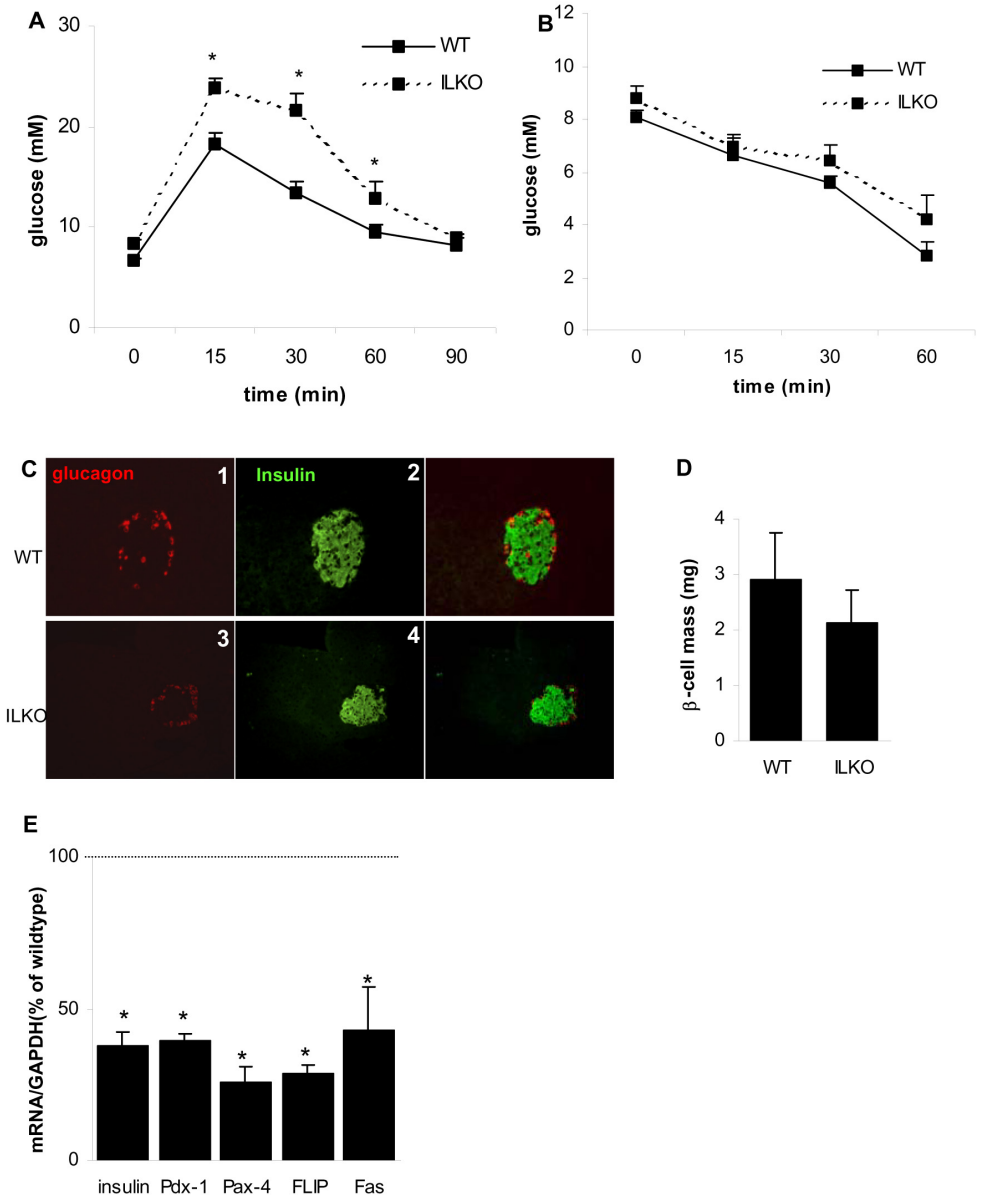


**Figure 3.4:** Impaired glucose tolerance in IL-1β knockout mice: Blood glucose levels following i.p. injection of glucose **(A)** or insulin **(B)** in 3 month old male IL-1β knockout (ILKO) and wildtype (WT) C57BL/6j mice. * indicates $p < 0.05$, ILKO versus WT. Data were collected from three separate experiments, each with five animals per group. **(C)** Double immunostaining for glucagon in red **(1, 3)** and insulin in green **(2, 4)** and **(D)** β-cell mass in tissue sections of 4 month old male WT **(1, 2)** and ILKO mice **(3, 4)**; $n = 5$ for each group. **(E)** Quantitative RT-PCR detection of insulin, PDX-1, Pax4, FLIP and Fas mRNA expression. Total RNA was isolated from ILKO and WT islets after 1 day in culture. The level of mRNA expression was normalized to GAPDH and the results expressed as percentage of WT islet mRNA levels; $n = 3$ for each group of mice islets, each in duplicate. * $p < 0.05$ to WT islets.

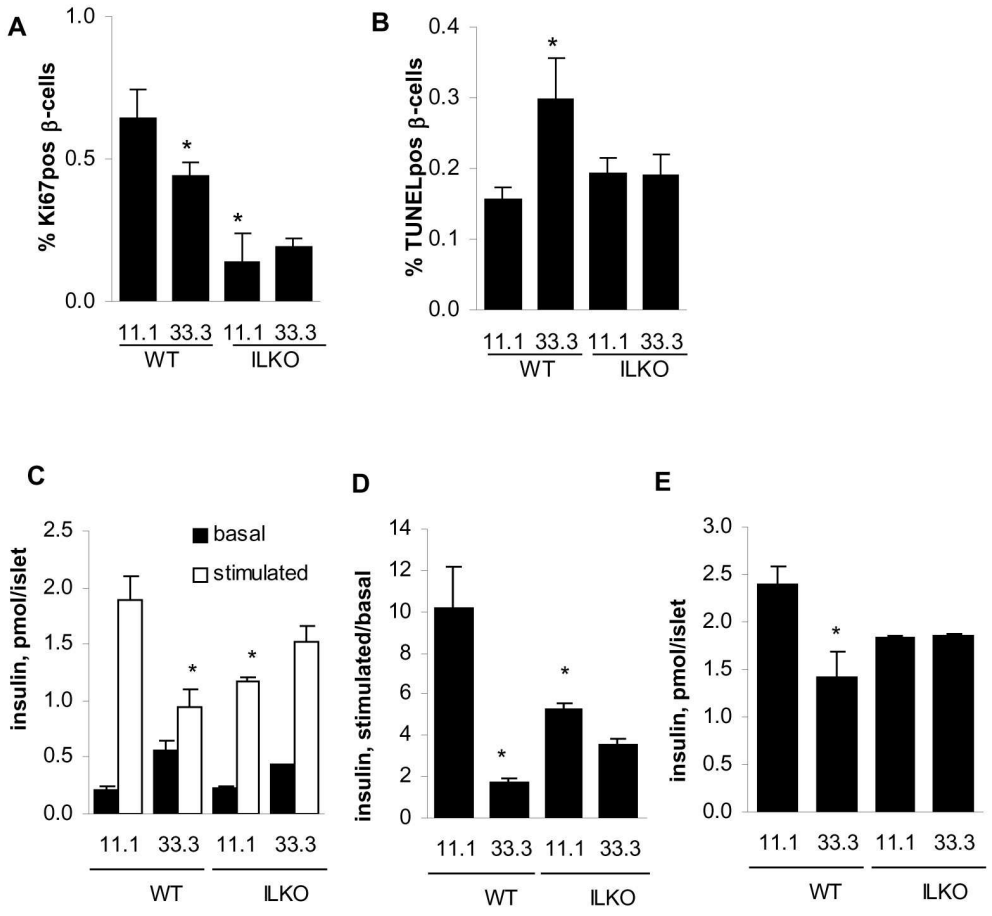


**Figure 3.5:** Resistance of IL-1$\beta^{-/-}$ islets to glucotoxicity: Isolated islets from male IL-1β knockout (ILKO) and wildtype (WT) mice were cultured on extracellular matrix-coated dishes for 4 days at 11.1 mM or 33.3 mM glucose. Results are means $\pm$ SE of percentage of Ki-67-positive **(A)** and TUNEL-positive **(B)** β-cells. The mean number of islets scored was 132 **(A)** and 118 **(B)** for each treatment condition in three independent experiments. **(C)** Basal and glucose-stimulated insulin secretion denote the amount secreted during successive 1-hour incubations at 2.8 mM (basal) and 16.7 mM (stimulated) glucose following the 4-day culture period expressed as secreted insulin **(C)** or stimulatory index **(D)**. **(E)** insulin content. Results are means $\pm$ SE. Islets were isolated from nine mice for each treatment group in three independent experiments. * $p < 0.05$ to WT mice.

both prevented IL-1β induced β-cell proliferation.

The physiological importance of IL-1β was also apparent in IL-1β knockout mice, which showed impaired glucose tolerance. Islets of these mice displayed decreased mRNA expression levels of PDX-1, Pax4, insulin, Fas and FLIP. Of note, these transcripts were enhanced by 0.02 ng/ml IL-1β. At higher concentrations, IL-1β suppressed these factors uncovering its toxic effects. IL-1β may participate in glucotoxicity [18], a concept substantiated by the protection from harmful actions of high glucose of IL-1β knockout mouse islets.

Most factors playing a role in β-cell turnover are also involved in the regulation of β-cell secretory function [57]. This is also true for IL-1β, which has distinct effects depending on the duration of exposure. On the short-term, IL-1β predominantly influenced β-cell secretory function independently of changes in β-cell mass. Indeed, in vitro, a 4-day exposure of human islets to different concentrations of IL-1β led to changes in cell turnover in less than 1% of β-cells although β-cell secretory function was almost completely blocked at high concentrations. Nevertheless, this does not mean that prolonged exposure to IL-1β will not affect β-cell mass. Indeed, β-cells remain positive for Ki-67 for approximately 12 hours. In absolute value, 0.02 ng/ml IL-1β increased the number of proliferating β-cells by 0.2%. The human pancreas has between 500,000 to 1,000,000 islets, comprising 500 to 2,000 β-cells each. Therefore, it can be estimated that low concentrations of IL-1β will increase the number of β-cells by approximately 4,000,000 per day, leading to a doubling of β-cell mass within 250 days. Such an impressive increase in β-cell mass may occur in vivo over a similar time period (e.g. during obesity [4]). Therefore, the observed differences are within an expected range and support the relevance of the finding.

A key signaling molecule regulating β-cell turnover and function is IRS-2 [58]. Interestingly, it has been proposed that IL-1β may mediate its deleterious effects via IRS-2 degradation [8]. Conversely, it is conceivable that at low concentrations IL-1β also signals via IRS-2. Indeed, while IRS-2 degradation leads to apoptosis, IRS-2 activation enhances β-cell proliferation and function. However, further investigations are required to support this hypothesis.

Cytokines are central in the development of diabetes. However, at low concentrations, IL-1β promotes β-cell function and survival. Therefore, glucose-induced IL-1β may have an important role in the long-term adaptation of the β-cells to hyperglycemia. Short term exposure to hyperglycemia will induce low levels of IL-1β, inducing IL-1Ra, Fas and FLIP, leading to decreased apoptosis and enhanced proliferation and function. However, prolonged hyperglycemia will increase the ratio of IL-1β to IL-1Ra decreasing FLIP and directing Fas to signals deleterious to the cell.

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# 4 A potential role for Fas and PDX-1 in age-related changes in β-cell turnover

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**Abstract**

Type 2 diabetes is characterized by a deficit in β-cell mass and its incidence increases with age. Here we analyzed β-cell turnover in islets from 2–3 compared to 7–8 month old rats and in human islets from 53 organ donors with ages ranging from 17 to 74 years. In cultured islets from 2–3-month old rats, the age at which rats are usually investigated, increasing glucose from 5.5 to 11.1 mM decreased β-cell apoptosis, which was augmented when glucose was further increased to 33.3 mM. In parallel, β-cell proliferation was increased by both 11.1 and 33.3 mM glucose compared to 5.5 mM. In contrast, in islets from 7–8 month old rats and from adult humans, increasing glucose concentrations from 5.5 to 33.3 mM induced a linear increase in β-cell death and a decrease in proliferation. Additionally, in cultivated human islets, age correlated positively with the sensitivity to glucose-induced β-cell apoptosis and negatively to baseline proliferation. In rat islets, constitutive expression of Fas-ligand (FasL) and glucose-induced Fas receptor expression were observed only in 7–8 month but not in 2–3 month old islets. Moreover, PDX-1 expression decreased with age in pancreatic tissue sections of rats and humans. Furthermore, older rat islets were more sensitive to the high glucose mediated decrease in PDX-1 expression than younger islets. Therefore, differences in glucose sensitivity between human and 2–3 month old rat islets may be due to age-dependent changes rather than to genetic background. These data provide a possible explanation for the increased incidence of type 2 diabetes at an older age and support the use of islets from older rats as a more appropriate model to study glucose-induced β-cell apoptosis.

## 4.1 Introduction

The prevalence of type 2 diabetes increases with age due to alteration or insufficient compensation of β-cell functional mass in the face of increasing insulin resistance [1–4]. While the existence of an inadequate adaptation of β-cell mass in patients with type 2 diabetes is beyond controversy [5–9], the effect of age on the sensitivity to pro-apoptotic stimuli of the human β-cell remains to be investigated.

Changes in glucose concentrations are key regulators of β-cell proliferation and apoptosis. Indeed, graded increases in glucose concentrations induce β-cell apoptosis in cultured islets from the diabetes-prone Psammomys obesus and from human islets [10–13]. In contrast, in islets from 2–3-month old rats, the age at which rats are usually investigated, an increase in glucose concentrations to 11.1 mM promotes β-cell survival [10, 14]. When glucose concentrations are further increased, glucose proves to be pro- or anti-apoptotic depending on culture conditions [10, 14, 15]. Investigations of β-cell proliferation revealed induction of proliferation by glucose in rat, Psammomys obesus and human islets [10, 11, 16]. Nevertheless, unlike the long-lasting effect in rat islets, only a transient and reduced proliferative response was observed in Psammomys obesus and human islets. Previous studies have shown that β-cell replication declines as rodents age and stabilizes at a rate of 1–3% per day [17–19] or even lower according to recent data [20]. However, β-cell turnover in aging humans remains unclear.

In human islets, the mechanism underlying glucose-induced β-cell apoptosis and impaired proliferation, involves the up-regulation of the Fas receptor, which interacts with the constitutively expressed Fas-ligand (FasL) on neighboring β-cells [11, 21]. In an interesting study, J. Hanke detected expression of FasL in β-cells of 6 month and older rats while FasL was not expressed in younger animals [22]. It is not known whether glucose-induced Fas is age-dependent.

Pancreatic duodenal homeobox-1 (PDX-1) is a β-cell specific transcription factor regulating β-cell differentiation and secretory function [23]. Furthermore, PDX-1 promotes β-cell replication and is cytoprotective [24–26]. Interestingly, in human pancreatic islets, PDX-1 may mediate deleterious effects of high glucose concentrations [27].

Therefore, we investigated the changes in β-cell turnover in relation to age. We show that age correlates with decreased proliferative activity and enhanced sensitivity to glucose-induced apoptosis. In parallel, constitutive expression of FasL and inducible Fas expression appeared along with decreased expression of PDX-1 under normal and gluco-toxic conditions.

## 4.2 Research design and methods

### 4.2.1 Islet isolation and culture

Human islets were isolated from pancreata of 53 organ donors. Islet purity was greater than 95% as judged by dithizone staining (if this degree of purity was not achieved by routine isolation, islets were hand-picked). The donors, aged 17–74 years, were heart-beating cadaver organ donors, and none had a previous history of diabetes or metabolic disorders. Male Sprague Dawley rats, aged 2–3 months (200–220 g) and 7–8 months (500–600 g), were anesthetized and islets isolated from the pancreata as previously described [28]. The islets were cultured on extracellular matrix-coated plates derived from bovine corneal endothelial cells (Novamed Ltd., Jerusalem, Israel), allowing the cells to attach to the dishes and spread. This mono- or bi-layer of cells has direct access to nutrients and oxygen allowing long-

term preservation of their functional integrity. Human islets were cultured in CMRL 1066 medium containing 5.5 mM glucose and rat islets were cultured in RPMI 1640 medium containing 11.1 mM glucose, both supplemented with 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 10% FCS (Invitrogen Ltd., Carlsbad, CA), hereafter referred to as culture medium. Two days after plating, when most islets were attached and had begun to flatten, the medium was changed to culture medium containing 5.5, 11.1 or 33.3 mM glucose for four days.

### 4.2.2 Detection of Fas, FasL and PDX-1 expressing β-cells

Pancreata from routine necropsies were immersion-fixed in formalin followed by paraffin embedding. Sections were deparaffinized and rehydrated and endogenous peroxidase blocked by submersion in 0.3% $H_2O_2$ for 15 min., after that, sections were incubated in methanol for 5 min and double stained with rabbit anti-PDX-1 antibody (kindly provided by Christopher Wright, Vanderbilt University Medical Center, Nashville) and insulin. Islet cultures were fixed in 4% paraformaldehyde (30 min, room temperature) followed by permeabilisation with 0.5% triton X-100 (4 min, room temperature) and were double-labeled for Fas receptor, FasL or PDX-1 by 1 h exposure to 10% bovine serum albumin, followed by incubation with rabbit anti-Fas (FL-335, Santa Cruz Biotech, Santa Cruz, CA), mouse anti-FasL (Transduction Laboratories, Lexington, KY), and detection using the streptavidin-biotin-peroxidase complex (Zymed, San Francisco, CA) or donkey anti-mouse Cy3 conjugated antibody (Jackson, Immunoresearch Laboratories, West Grove, PA). Subsequently, the specimens were stained for insulin as previously described [10, 11]. The specificity of the Fas and FasL antibodies was assessed previously [11]. Saturation of staining was analyzed using analySISTM 3.1 software (Soft Imaging System GmbH, Muenster, Germany).

### 4.2.3 Beta-cell replication and apoptosis

For β-cell replication and apoptosis studies, islet cells were double stained with either a monoclonal antibody against the human or rat Ki-67 antigen (Zymed) or by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) according to the manufacturer's instructions (In Situ Cell Death Detection Kit, AP; Boehringer Mannheim, Germany) and for insulin as described above.

### 4.2.4 Western blot analysis

Islets were cultured in suspension in RPMI 1640 (rat) or CMRL 1066 (human) medium as described above. Equivalent amounts of protein from each treatment group were run on 15% SDS polyacrylamide gels as described previously [29]. Proteins were electrically transferred to nitrocellulose filters and incubated with rabbit anti-Fas antibody (FL-335, Santa Cruz Biotech, Santa Cruz, CA), mouse anti-FasL (Transduction Laboratories), or with mouse anti-PDX-1 (kindly provided by Stefan Zahn, Novo Nordisk A/S, Bagsvrd, Denmark) or rabbit anti-actin (Cell signaling Technology, MA, USA) antibodies followed by incubation with horseradish-

peroxidase-linked anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology Inc.). Immune complexes were detected by chemiluminescence using LumiGLO (Cell Signalling).

### 4.2.5 RNA extraction and quantitative RT-PCR

Total RNA was extracted from the cultured islets by using the RNeasy mini kit (Qiagen Inc., Basel, Switzerland) and RT-PCR was performed using the SuperScript Double-Stranded cDNA synthesis kit according to the manufacturer's instructions (Life Technologies, Gibco, Gaithersburg, MD). For quantitative analysis, we used the LightCycler quantitative PCR system (Roche, Basel, Switzerland) with a commercial kit (Light Cycler-DNA Master SYBR Green I; Roche). The primers were 5′CCACCTTGGGACCTGTTTAG3′ and 5′TGATGCCAGAGGAAGAGGAG3′ (human PDX-1); 5′GAGGACCCGTACAGCCTACA3′ and 5′CGTTGTCCCGCTACTACGTT3′ (rat PDX-1); 5′TTCTTCTACACACCCA3′ and 5′CTAGTTGCAGTAGTTCT3′ (insulin) 5′AGAGTCGCGCTGTAAGAAGC3′ and 5′TGGTCTTGTCACTTGGCATC3′ ($\alpha$-Tubulin); 5′AACAGCGACACCCACTCCTC3′ and 5′GGAGGGGAGATTCAGTGTGGT3′ (GAPDH).

### 4.2.6 Glucose stimulated insulin secretion

For acute insulin release in response to glucose, islets were washed and pre-incubated (30 min) in Kreb's Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose and 0.5% BSA. KRB was then replaced by KRB 2.8 mM glucose for 1 h (basal), followed by an additional 1 h in KRB 16.7 mM glucose. Islets were extracted with 0.18 N HCl in 70% ethanol for determination of insulin content. Insulin was determined using a human insulin RIA kit (CIS bio international, Gif-Sur-Yvette, France), which has similar affinity for both rat and human insulin.

### 4.2.7 Evaluation and statistical analysis

Samples were evaluated in a randomised manner by a single investigator (K. M.) blind to the treatment conditions. Data were analysed by Student's t-test, or by analysis of variance with a Bonferroni correction for multiple group comparisons, or by regression analysis using GraphPad Prism, version 3.0 (San Diego, CA, USA).

## 4.3 Results

### 4.3.1 Distinct effects of glucose-induced changes in β-cell apoptosis and proliferation are not species- but age-dependent

Analysis of human islets cultured for 4 days at 11.1 and 33.3 mM glucose revealed a 2.0- and 2.8-fold increase in β-cell nuclei with DNA fragmentation (TUNEL-positive, Fig. 4.1 A), respectively, relative to islets at 5.5 mM glucose. In parallel,

β-cell proliferation was 1.5-fold and 2.7-fold reduced by 11.1 and 33.3 mM glucose, respectively (Fig. 4.1 B). In contrast, exposure of 2–3 month old rat islets to increasing glucose concentrations resulted in an inverse bell-shaped response. The lowest incidence of apoptotic β-cell nuclei occurred at a glucose concentration of 11.1 mM and increased 3.6- and 3.0-fold at 5.5 mM and 33.3 mM glucose, respectively (Fig. 4.1 C & G). Proliferation of 2–3 month old rat β-cells was increased by 1.9- and 1.8-fold at 11.1 and 33.3 mM glucose, respectively, as compared to islets at 5.5 mM glucose (Fig. 4.1 D & G). However, islets from 7–8 month old rats behaved similarly to human islets, with a progressive induction of β-cell apoptosis of 1.3- and 2.9-fold by 11.1 mM and 33.3 mM glucose , respectively, and a 1.4-fold decrease of β-cell proliferation at 33.3 mM glucose (Fig. 4.1 E–G).

### 4.3.2 Aging correlates with enhanced sensitivity to glucose-induced β-cell apoptosis and decreased baseline proliferation

Since the incidence of diabetes increases with age, we were interested to know whether this correlates with changes in the proliferative capacity and in the sensitivity to glucose-induced apoptosis of β-cells. Therefore we analyzed baseline and glucose-stimulated apoptosis and proliferation rates of β-cells from cultivated islets from 53 organ donors with ages ranging from 17 to 74 years. Mean baseline β-cell apoptosis at 5.5 mM glucose after four days of culture was 0.43% TUNEL positive β-cells and did not significantly change with age (Fig. 4.2 A). However, there was a significant correlation between the age of the donor and the sensitivity to glucose-induced apoptosis (Fig. 4.2 B). In contrast, the baseline proliferation rate of cultured human β-cells was negatively correlated with increasing age of the donor whereas the deleterious effect of glucose on the replicating rate of β-cells was age-independent (Fig. 4.2 C & D).

### 4.3.3 Age-dependent appearance of the Fas/FasL system

In human islets, the mechanism underlying glucose-induced β-cell apoptosis and impaired proliferation involves the up-regulation of Fas, which interacts with constitutively expressed FasL on neighboring β-cells [11]. Therefore, we hypothesized that age-dependent changes in Fas and FasL expression could explain differences in β-cellapoptosis and proliferation seen in rats of different ages. Double-immunostaining of cultivated islets from 2–3 month old rats revealed neither expression of the Fas receptor nor of FasL at 5.5 mM and 33.3 mM glucose (Fig. 4.3 A, panels 1–8). In contrast, β-cells from 7–8 month old rats constitutively expressed FasL at low and high glucose concentrations (Fig. 3 A, panels 9–12). Moreover, similar to human β-cells [11], glucose induced expression of Fas at 11.1 mM and 33.3 mM glucose concentrations (Fig. 4.3 A, panels 15 & 16), compared to 5.5 mM (Fig. 4.3 A, panels 13 & 14). Age-dependent Fas receptor and FasL expression was confirmed by Western Blot analysis of lysates from 2–3 and 7–8 month old rat islets cultured in suspension at 5.5, 11.1 and 33.3 mM glucose (Fig. 4.3 B). After 30 and 96 h of cul-

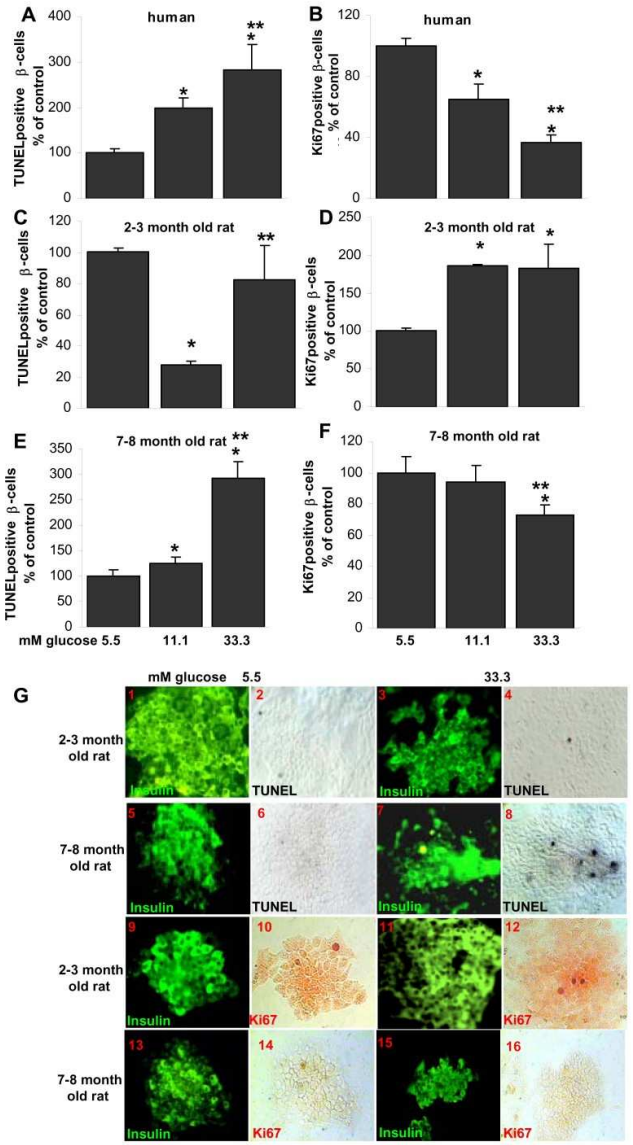


**Figure 4.1:** Response to glucose-induced changes in β-cell apoptosis and proliferation are age-dependent. Human **(A, B)**, 2–3 month **(C, D & G panels 1–4 & 9–12)** and 7–8 month old rat **(E, F & G panels 5–8 & 13–16)** pancreatic islets were cultured on extracellular matrix-coated dishes, exposed for 4 days to media containing 5.5, 11.1 or 33.3 mM glucose and double-immunostained for insulin and DNA fragmentation by the TUNEL assay **(A, C, E & G panels 1–8)** or for proliferation by the Ki-67 antibody **(B, D, F & G panels 9–16)**. Results are means ± SE of the relative percentage of TUNEL-positive or Ki-67-positive β-cells relative to control incubations at 5.5 mM glucose alone (100%; in absolute values: **A**: 0.43% TUNEL positive β-cells, **B**: 0.53% Ki67-positive β-cells, **C**: 0.82% TUNEL positive β-cells, **D**: 2.06% Ki67-positive β-cells, **E**: 1.10% TUNEL positive β-cells and **F**: 0.83% Ki67-positive β-cells at 5.5 mM glucose alone). The mean number of islets scored was **A**: 81, **B**: 74, **C**: 65, **D**: 55, **E**: 71, **F**: 76 for each treatment condition from each donor. Islets were isolated from seven human organ donors and from 32 rats aged 2–3 and 7–8 months. * $p < 0.05$ relative to islets at 5.5 mM glucose, ** $p < 0.05$ relative to islets at 11.1 mM glucose.

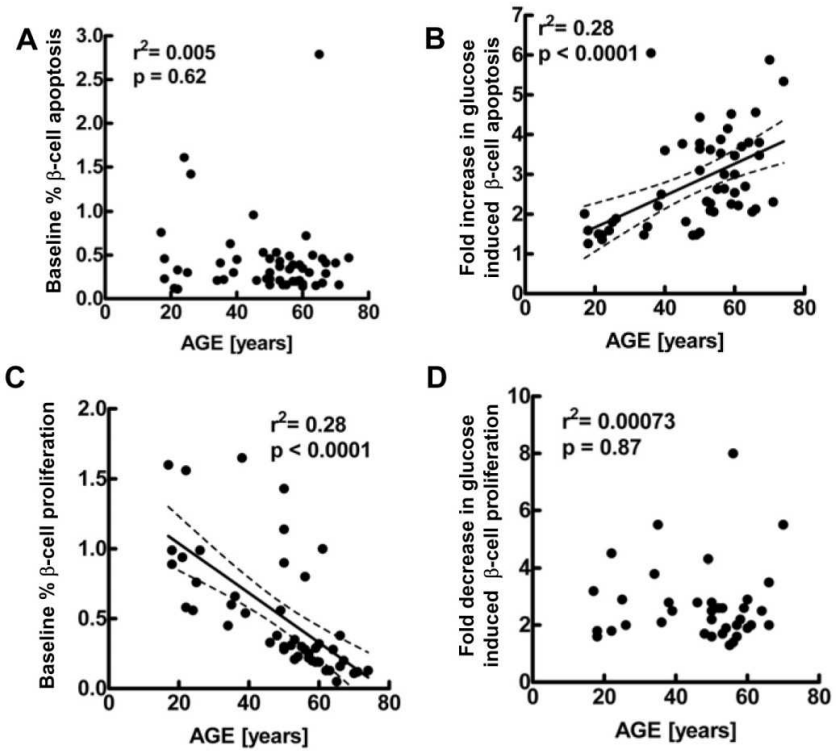


**Figure 4.2:** Aging correlates with enhanced sensitivity to glucose-induced β-cell apoptosis and decreased baseline proliferation. Human pancreatic islets were isolated from 53 organ donors of ages ranging from 17 to 74 years and cultured on extracellular matrix-coated dishes, exposed for four days to media containing 5.5 or 33.3 mM glucose and double-immunostained for insulin and the TUNEL assay **(A & B)** or the Ki-67 antibody **(C & D)**. Correlation between age and means of the percentage of TUNEL-positive or Ki-67-positive β-cells at baseline 5.5 mM glucose **(A & C)** or at 33.3 mM glucose relative to control incubations at 5.5 mM glucose alone. Each datum point represents an independent experiment of one organ donor. Solid lines are best fit, with broken lines showing 95% Cis.

ture, FasL was present in the 7–8 month old rats at all glucose concentrations, but not in the 2–3 month old rats. Fas receptor was up-regulated in the 7–8 month old rats by 11.1 and 33.3 compared to 5.5 mM glucose after 30 h, and at higher levels after 96 h of culture. Finally, Fas receptor was almost undetectable in the islets of 2–3 month old rats cultured at low or high glucose concentrations for 30 or 96 h. In contrast, in human islets, FasL was already present at 5 days of age, and no changes in expression levels could be detected with aging (Fig. 4.3 C). We also performed analysis for the Fas receptor in humans at different ages from 21 to 71 years. Increasing glucose concentrations induced Fas receptor up-regulation, but no age dependent differences could be quantified (Fig. 4.3 D). In parallel, β-cell secretory function was analyzed. Glucose stimulated insulin secretion decreased with age in rats as well as in humans (Fig. 4.3 E).

### 4.3.4 Beta-cell expression of PDX-1 decreases with age

We next studied the influence of aging on PDX-1 expression under normal and glucotoxic conditions. Beta-cell expression of PDX-1 was strongly decreased in islets of 7–8 month old rats as compared to younger rats. This was observed by Western Blotting of cultured islet lysates (Fig. 4.4 A), quantitative RT-PCR (Fig. 4.4 B) and by immunostaining of tissue sections (Fig. 4.4 C & D). Exposure of the islets to increasing glucose concentrations for 30 and 96 h decreased PDX-1 expression in 7–8 month old islets whereas in 2–3 month old islets a decrease occurred only after prolonged culture for 96 h but not after 30 h (Fig. 4.4 A). Subsequently we analyzed tissue sections from necropsies of 18 human pancreata from non-diabetic individuals from 5 days to 79 years old. PDX-1 was clearly expressed in β-cells of young individuals whereas it was barely detectable in pancreata of old individuals (for representative images see Fig. 4.4 E). Quantitative analysis of the intensity of the PDX-1 staining revealed a significant negative correlation between the age of the patients and PDX-1 expression (Fig. 4.4 F). Finally, PDX-1 mRNA expression levels from 23 human islet isolations were studied and also revealed a negative correlation with age, both compared to tubulin (Fig. 4.4 G) or GAPDH.

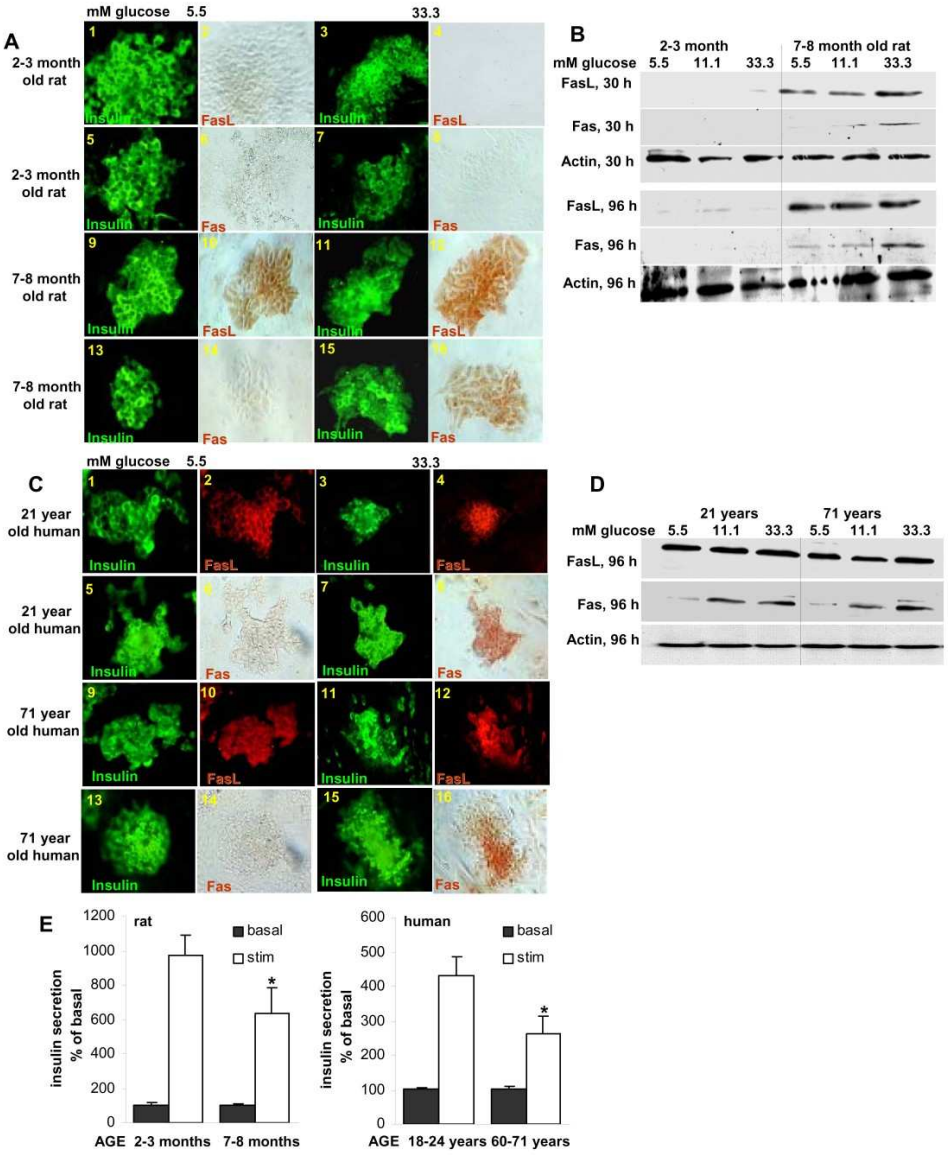


**Figure 4.3:** Age-dependent appearance of the Fas/FasL system. Pancreatic islets isolated from 2 month **(A panels 1–8 & B)** and 8 month **(A panels 9–16 & B)** old rats, and from 21 year **(C panels 1–8 & D)** and 71 year old humans **(C panels 9–16 & D)** were cultured on extracellular matrix coated dishes **(A & C)** or in suspension **(B & D)**, and exposed for 30–96 h to media containing 5.5, 11.1 or 33.3 mM glucose. Double-immunostaining for insulin in green **(A & C odd-numbered panels)** and FasL **(A & C panels 2, 4, 10, 12)** or the Fas receptor in red **(A & C panels 6, 8, 14, 16)**. **(B & D)**: Western Blot analysis of FasL, Fas and actin. The antibodies were blotted on the same membrane after stripping. One representative of three experiments from isolated islets from 2–3 and 7–8 month old rats and from 18–21 and 60–71 year old human organ donors is shown, respectively. **(E)** In parallel, β-cell secretion assays from the isolated rat and human islets were performed. Basal and glucose-stimulated insulin secretion denote the amount secreted during successive 1-hour incubations at 2.8 mM (basal) and 16.7 mM (stimulated) glucose, normalised to insulin content and expressed as percent change from basal secretion. Islets were isolated from four 2–3 month old and four 7–8 month old rats, and from four 18–24 year old and four 60–71 year old human organ donors and plated in four dishes per experiment. * $p < 0.05$ relative to stimulated insulin secretion from 2–3 month old rats and 18–24 year old humans, respectively.

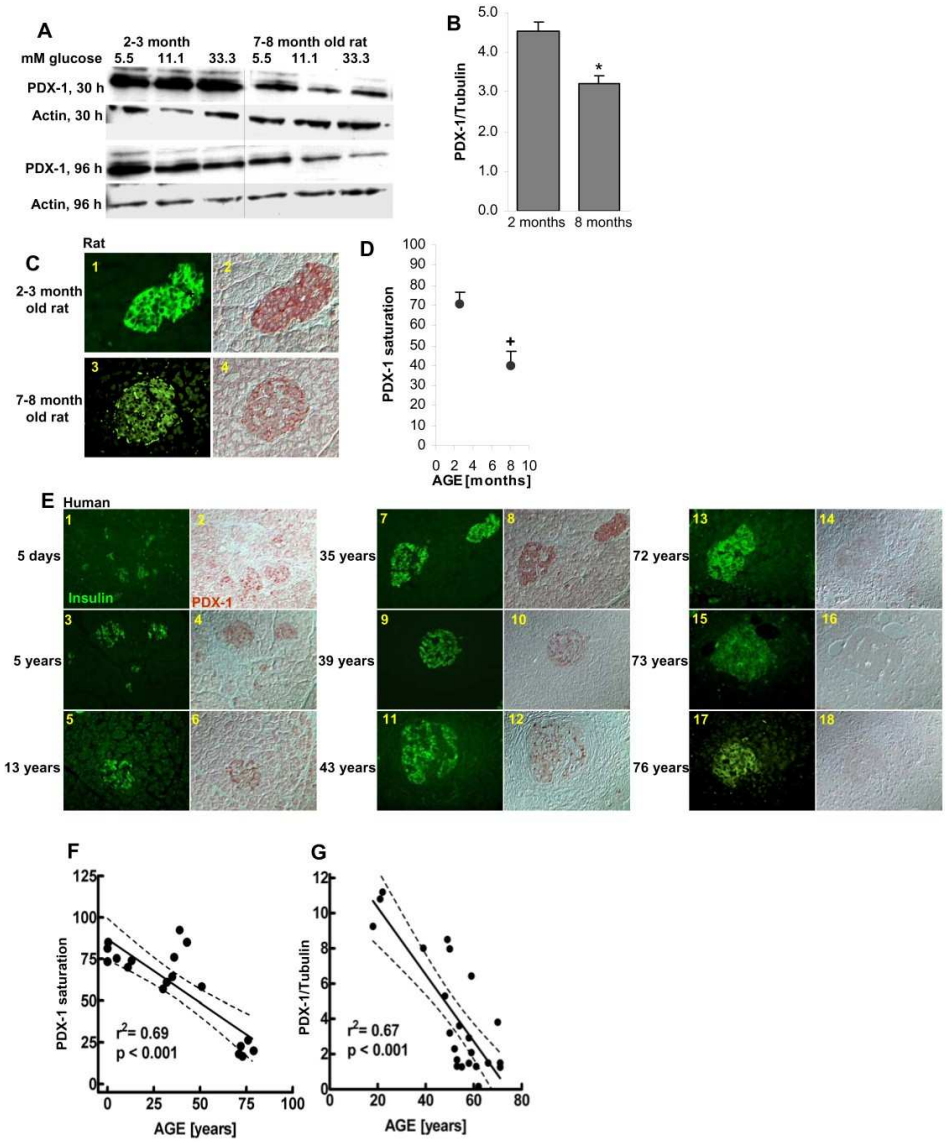


**Figure 4.4:** Beta-cell expression of PDX-1 decreases with age. **(A)** Pancreatic islets isolated from 2–3 and 7–8 month old rats were cultured in suspension and exposed for 30 or 96 h to media containing 5.5, 11.1 or 33.3 mM glucose. Western Blot analysis of PDX-1 and actin. The antibodies were blotted on the same membrane after stripping. One of three experiments is shown. **(B)** RT-PCR quantification of PDX-1 mRNA expression in total RNA isolated from 2 and 8 month old rat islets following overnight incubation at 11.1 mM glucose. The levels of PDX-1 expression were normalized against tubulin. Double-immunostaining for PDX-1 in red **(C panels 2, 4 & E even-numbered panels)** and insulin in green **(C panels 1, 3 & E odd-numbered panels)** on pancreatic sections from 2–3 and 7–8 month old rats **(C & D)** and from 5 days, 5, 13, 35, 39, 43, 72, 73 and 76 year old humans **(E & F)**. Intensity of PDX-1 immunostaining relative to background in β-cells identified by doublestaining for insulin in rat **(D)** and human **(F)** sections. In **(D)** results are shown as mean from five rats ± SE and in **(F)** as a correlation between PDX-1 and age from 18 human pancreas sections. Ten islets were scored in each section. + $p < 0.05$ relative to islets at 2 months of age. **(G)** Correlation between age and RT-PCR quantification of PDX-1 mRNA expression in total RNA isolated from 23 human islet isolations. The level of PDX-1 expression was normalized against tubulin. Each datum point represents an independent experiment of one organ donor. Solid lines are best fit, with broken lines showing 95% Cis.

## 4.4 Discussion

Aging is a major risk factor for the development of type 2 diabetes. In cultivated human islets, aging was negatively correlated with baseline β-cell proliferative activity, and positively correlated with enhanced sensitivity to glucose-induced apoptosis. These in vitro observations are supported by Butler et al who show a trend for decreased β-cell replication with age [6]. Thus, the limited adaptive capacity of aging β-cells may contribute to the risk observed in elderly patients to develop type 2 diabetes.

Many differences exist between rat and human islets which are usually attributed to differences in the genetic background. Indeed, islets from 2–3 month old rats and human islets responded differently to glucose-induced changes in cell turnover. However, while 2–3 month old rats are often considered to be adult, this is certainly not the case in many aspects, including linear growth. No striking cell cycle differences were apparent when comparing human islets and islets from rats older than 6 months. This can be explained partly by the appearance of the Fas/FasL system. Indeed, FasL is constitutively expressed by β-cells from rats aged 6 months and older but not in younger rats, as previously shown [22] and confirmed in the present study. Similarly, glucose induced Fas expression only in β-cells from 7–8 month old rats and not at 2–3 months. Therefore, some differences between human islets, usually emanating from adult organ donors, and rodent islets may be due to differences in age and not in genetic background. However, we cannot rule out an intrinsic genetic difference in the proliferative capacity between the species.

In human islets, baseline β-cell proliferation decreased with age. Furthermore, prolonged exposure to 33.3 mM glucose decreased β-cell proliferation of cultured islets from 7–8 month old rats and from adult humans. In parallel, both aging and prolonged exposure to glucose were associated with decreased expression of PDX-1. Interestingly, islets from 2–3 month old rats displayed a reduced expression of PDX-1 only following a 96 h-exposure to high glucose but not after 30 h. Therefore, changes in PDX-1 activity might contribute to these changes in proliferation rates. Supporting this notion, PDX-1 has been shown to be important for β-cell replicative activity and survival [24–26]. Furthermore, chronic exposure of cultured human pancreatic islets to high glucose lowers the activity of PDX-1 [27]. Yet, our data are correlative and a causative proof remains to be shown.

The observed changes in β-cell turnover most probably did not result in significant changes in β-cell area in vitro. Indeed, a 4-day exposure of islets to elevated glucose concentrations induced changes in apoptosis and proliferation in only 1–2% of the β-cells. However, these findings may be relevant for the in vivo situation. Indeed, a net increase of 1% in cell turnover will lead to a doubling in β-cell mass whereas a net decrease of 1% will lead to a 50% decrease in β-cell mass, within 3–4 months. Such impressive changes in β-cell mass may occur in vivo over a similar time period (e.g. during obesity, see [6]). Nevertheless, in parallel to changes in β-cell turnover, aging is associated with a progressive decrease in β-cell function, as previously described [2, 30] and confirmed in the present study.

In conclusion, we demonstrate that there is a progressive impairment in β-cell turnover with age, characterised by decreased baseline proliferation and enhanced

sensitivity to glucose-induced β-cell apoptosis. This impairment is associated with decreased PDX-1 expression. Furthermore, in rat islets, appearance with age of the Fas/FasL pathway parallels changes in sensitivity to glucose-induced apoptosis and decreased proliferation. Therefore, changes in β-cell plasticity may be a predisposing factor to the development of diabetes in elderly subjects. Finally, islets of older rats may be a more appropriate model than younger islets to study glucose-induced β-cell apoptosis.

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# 5 Fas deficient mice are protected from High-Fat diet induced β-cell function impairment.

**Abstract**

We have previously shown that the Fas receptor is needed for normal insulin secretion. Here, we examined the role of Fas during the development of diabetes. Wildtype and Fas deficient mice were fed a high fat diet for 4 weeks, after which, the wildtype mice developed impaired glucose tolerance and insulin resistance. Surprisingly, the Fas deficient mice, which exhibited impaired glucose tolerance when fed normal chow, showed an improved glucose tolerance when fed a high fat diet and no insulin resistance. Islets isolated from these animals, showed that the Fas deficient mice on the high fat diet, had a significantly improved glucose-stimulated insulin secretion, and an increase in insulin, Pdx-1, c-myc and Pax4 mRNA levels, compared to Fas deficient mice on the normal chow. Wildtype mice on a high fat diet had a decrease in all these genes. UCP-2 was increased in the wildtype mice but not in the Fas deficient mice on the high fat diet. This was confirmed in wildtype and Fas deficient islets exposed to palmitic/oleic acid. Similarly, human islets cultured in the presence of an antagonistic anti-Fas antibody displayed impaired glucose-induced insulin secretion which was reversed when treated with fatty acids. The glucotoxic effects of glucose on wildtype islets were reversed on transfection with FLICE inhibitory protein (FLIP). FLIP was able to improve glucose-induced insulin secretion, by increasing insulin and PDX-1 mRNA levels. Therefore, modulation of the Fas/FLIP pathway may prevent the development of type 2 diabetes.

## 5.1 Introduction

In humans, most obese individuals are able to adapt to their obesity and the resultant insulin resistance by increasing β-cell mass and insulin secretion. In those individuals that are unable to adapt, the β-cell secretes less insulin and glucose homeostasis is disrupted, resulting in hyperglycaemia and eventually type 2 diabetes. Impaired beta cell function and loss of beta cell mass are therefore central to the development of type 2 diabetes [1]. Hyperglycaemic conditions induce Fas in human islets which results in increased β-cell apoptosis and decreased β-cell function [2–4]. The Fas receptor, which is a member of the TNF superfamily, has long been recognized as

a death receptor, but recent evidence has shown that the Fas receptor is directly involved in proliferation [5, 6] and insulin secretion via the FLICE inhibitory protein (FLIP, as detailed in Chapter 2). FLIP is an anti-apoptotic protein that blocks the activation of procaspase-8 to caspase-8. In this study we sought to determine the importance of the Fas/FLIP pathway in the development of type 2 diabetes by placing Fas deficient mice on a high energy diet for 4 weeks. We then looked at β-cell function both in vivo and in vitro. The experiments were repeated in human islets and the Fas receptor was blocked with an antagonistic anti-Fas antibody, ZB4.

## 5.2 Methods

### 5.2.1 Animal maintenance

Ethical approval for mouse studies was granted by the Zürich Cantonal Animal Experimentation Committee. C57BL/6j (wildtype) and mice with a natural Fas mutation backcrossed for more than 10 generations onto this same C57BL/6j inbred strain background (B6.MRL-Tnfrsf6$^{lpr}$, Fas deficient) were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed at 22°C with a 12-h light-dark cycle (lights on at 07:00) and allowed free access to water and food. The wildtype and Fas deficient mice were divided into 4 groups. Two groups were placed on a normal Purina chow diet (Provimi Kliba AG, Kaiseraugst) and the other two groups were placed on a high energy diet (HFD, Research Diets, New Brunswick, NJ). The normal chow contained 29% fat compared to the HFD which contained 58% fat.

### 5.2.2 Islet isolation and culture

Pancreatic islets were isolated from wildtype and Fas deficient mice using Collagenase and an adapted protocol from Gotoh et al. [7]. After isolation, islets were cultured on extracellular matrix-coated plates derived from bovine corneal endothelial cells (Novamed Ltd., Jerusalem, Israel), and allowed to form a monolayer [8]. Islets were cultured in RPMI 1640 medium (hereafter referred to as culture medium) containing 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, Fungizone, Gentamycin and 10% FCS (Invitrogen Ltd., Carlsbad, California). One day after plating the islets, the medium was changed to culture medium containing 11.1 or 33.3 mM glucose. In some experiments, the islets were transfected with either a RIP-only vector or with a Rip-vector containing human FLIP cDNA.

### 5.2.3 Liposome-mediated transfection of mouse islets

Lipofectamine 2000-DNA complexes were prepared according to the manufacturer's instructions (Invitrogen Ltd., Carlsbad, California) using a RIP-FLAG-tagged FLIP long construct or a RIP-only vector. The RIP-FLAG-tagged FLIP long vector was constructed by excising FLAG-tagged FLIP from pCR3 using SmaI and XbaI. The FLAG-tagged FLIP was inserted into a vector containing 640 bp rat insulin promotor (RIP, kindly provided by T. Trueb, Zuerich, Switzerland). The solution was

added to the islets at a final concentration of 3 $\mu$g of DNA/ml in Optimem. After 6 hours incubation, 2 ml culture medium was added, and after 24 hours, the medium was aspirated and replaced with fresh culture medium according to the experimental conditions needed.

### 5.2.4 Histochemical analysis

Beta-cell proliferation was detected with Ki-67 an affinity- purified goat polyclonal antibody raised against mouse (Santa Cruz Biotechnology). Ki-67 is a nuclear antigen expressed by proliferating cells and is used as a marker for late G1, S, G2, and M phases of the cell cycle [9]. After washing with phosphate-buffered saline (PBS), the islets were fixed in 4% paraformaldehyde for 30 minutes at room temperature, followed by permeabilization with 0.5% Triton X-100 for 4 minutes at room temperature. The cells were then incubated for 1 hour at room temperature with the primary antibody diluted 1:50, followed by a PBS wash and then incubation with the secondary antibody, donkey-anti-goat (Santa Cruz Biotechnology). To visualise the beta-cells, we double-stained for insulin by incubating the islets for 30 minutes at 37°C with guinea pig anti-insulin antibody diluted 1:50 (Dako, Carpinteria, California), followed by a 30 minute incubation with a fluorescein-conjugated rabbit anti-guinea pig antibody (1:15, Dako, Carpinteria, California). Free 3-OH strand breaks, which are a result of DNA degradation, were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique. The TUNEL assay thus allows one to quantify apoptosis. Whole pancreas tissue was also sectioned to determine islet morphology. Islets were double-stained with insulin and glucagon and thereafter surface area was measured using the analySISTM 3.1 software (Soft Imaging System GmbH, Muenster, Germany) program to determine islet size.

### 5.2.5 RNA extraction and Quantitative Reverse Transcription-PCR (RT-PCR)

Total RNA was extracted from the cultured islets by using the Rneasy mini kit (Qiagen, Basel, Switzerland) and reverse transcription was performed using the SuperScript Double-Stranded cDNA synthesis kit according to the manufacturer's instructions (Life Technologies, Invitrogen). For quantitative analysis, we used the Light Cycler quantitative PCR system (Roche, Basel, Switzerland) and performed quantitative PCR with a commercial kit, Light Cycler-DNA Master SYBR Green I (Roche, Basel, Switzerland). We measured insulin, PDX-1, c-myc, Pax4, UCP-2 and human FLIP mRNA using the following primers: insulin – 5′ TACGGGGTTTGTGAAAGGAG 3′ and 5′ CACATCATTCCCCAGGAAAC 3′; PDX-1 – 5′ GAGGACCCGTACAGCCTACA 3′ and 5′ CGTTGTCCCGCTACTACGTT 3′; c-myc – 5′ TCTCCACTCACCAGCACAAC 3′ and 5′ AATTCAGGGATCTGGTCACG 3′; Pax4 – 5′ GCTCTTTTTGCCTGGGAGATC 3′ and 5′ CCCGAAGGACTCGATTGATAGA 3′ UCP-2 – 5′ CAGCCAGCGCCCAGTACC 3′ and 5′ CAATGCGGACGGAGGCAAAGC 3′ and human FLIP – 5′ CTAAATTTGGTTGCCCCAGA 3′ and 5′ CTCC-

CATTATGGAGCCTGAA 3′.

### 5.2.6 Insulin secretion and content

To determine acute glucose stimulated insulin release, cultured islets were preincubated in Krebs Ringer Buffer containing 3.3 mM glucose for 30 minutes in at 37∘C in air. This medium was aspirated and the islets re-incubated in Krebs Ringer buffer containing 3.3 mM glucose for 1 hour at 37°C in air. This was to determine basal insulin secretion. Thereafter, the islets were incubated in Krebs Ringer buffer containing 16.7 mM glucose for 1 hour at 37°C in air. For insulin content, the islets were exposed to 0.18 N HCl in 70% ethanol for 24 hours at 4°C. Insulin was measured using a human insulin RIA kit (CIS Biointernational, Gif-Sur-Yvette, France) which was tested and shown to cross-react with mouse insulin.

### 5.2.7 Intra-peritoneal glucose tolerance test (IPGTT) and intra-peritoneal insulin tolerance test (IPITT)

An IPGTT was performed to characterize the glucose tolerance of Fas-deficient mice compared to wildtype mice. The mice were maintained on a either a normal chow or high fat diet (HFD) and water ad libitum before being fasted overnight (12 hours), and then injected intraperitoneally with 40% glucose solution (2 mg/g body weight, Glucosum, Dr. G. Bichsel). Glucose was administered with a 0.5 ml (U-100) insulin syringe (Becton Dickinson AG, Basel, Switzerland) through a 29 gauge needle. Blood glucose concentration was measured before (0 min) and 15, 30, 60, and 90 minutes after glucose injection with a Freestyle glucometer (Disetronic Medical Systems, Burgdorf, Switzerland). An intraperitoneal insulin tolerance test was performed on overnight fasted animals (12 hours). Blood glucose was measured at time 0 before insulin injection and 15, 30 and 60 minutes after insulin injection (0.75 U/kg Actrapid, Novo Nordisk, Copenhagen, Denmark).

### 5.2.8 In situ pancreas perfusion

Wildtype and Fas deficient mice aged 7-8 weeks, were anaesthetised with sodium pentothal 100 mg/kg bodyweight intraperitoneally and prepared for pancreas perfusion as previously described [10]. The pancreas was perfused at 37°C with modified Krebs Ringer HEPES buffer supplemented with the indicated glucose concentrations. The perfusion was maintained at 1 ml/min through the aorta into the pancreas. The pancreatic effluent of the first 30 minutes of perfusion with basal glucose (2.8 mM) was discarded. After this equilibration period, the effluent was collected in 1 min fractions from a catheter placed in the portal vein. The insulin content of each fraction was determined by radioimmunoassay.

## 5.3 Results

### High fat diet improves insulin secretion without development of insulin resistance in Fas deficient mice

Wildtype and Fas deficient mice on HFD gained a significant amount of weight during the treatment compared to the wildtype and Fas deficient mice on normal chow (Fig. 5.1 a). There were no differences in fasting blood glucose concentrations for wildtype and Fas deficient mice on the normal chow diet (Fig. 5.1 b). HFD wildtype mice significantly increased their fasting blood glucose compared with normal chow wildtype mice, but this increase did not occur in the Fas deficient mice (Fig. 5.1 b).

After a high fat diet, wildtype mice showed impaired glucose tolerance compared to wildtype mice on normal chow. Interestingly, Fas deficient mice on high fat diet had significantly improved glucose tolerance compared to Fas deficient mice on the normal chow diet. Insulin tolerance test showed no difference between wildtype and Fas deficient mice on normal chow (Fig. 5.1 d). Surprisingly, unlike the wildtype mice on high fat diet, the Fas deficient mice on a high fat diet showed no insulin resistance. This was confirmed with a glucose uptake test on adipocytes (Fig. 5.1 e).

In situ pancreatic perfusions showed a 2-minute delay in glucose stimulated insulin secretion by Fas-deficient mice on normal chow as well as a blunted glucose stimulated insulin secretion compared to wildtype mice on normal chow (Fig. 5.1 f). After a high fat diet, the Fas deficient mice showed normal glucose stimulated insulin secretion, with clear first and second phase insulin secretion (Fig. 5.1 f).

To determine the effects of the high fat diet on β-cell morphology and mass, we looked at pancreatic sections from Fas deficient and wildtype mice. The distribution of β- and $\alpha$-cells was normal in the Fas-deficient islets compared to the wildtype islets for both diets. Islets from wildtype mice on high fat diet showed a not significant increase in size, compared to normal chow wildtype mice whereas islets from Fas deficient mice on high fat diet showed a not significant decrease in size, compared to normal chow Fas deficient mice (Fig. 5.1 g).

Since there were no significant changes in β-cell mass, we decided to have a closer look at the islets. Islets from wildtype mice on the high fat diet had a significant decrease in glucose-induced insulin secretion compared to islets from wildtype mice on normal chow (Fig. 5.1 h). As previously shown in Chapter 2, Fas deficient mice on normal chow have a significant decrease in glucose stimulated insulin secretion compared to wildtype mice on normal chow. After the high fat diet, islets from Fas deficient mice showed a significant improvement in glucose-induced insulin secretion (Fig. 5.1 h). Gene expression analysis yielded a significant increase in insulin, PDX-1, c-myc and Pax4 mRNA in islets from Fas deficient mice on a high fat diet, compared to Fas deficient mice on a normal chow diet, whereas there is a significant decrease in these genes in islets from wildtype mice on a high fat diet (Fig. 5.1 i). UCP2 was significantly increased in the islets from wildtype mice on a high fat diet compared to islets from wildtype mice on a normal chow diet, but stayed unchanged in the Fas deficient islets (Fig. 5.1 i).

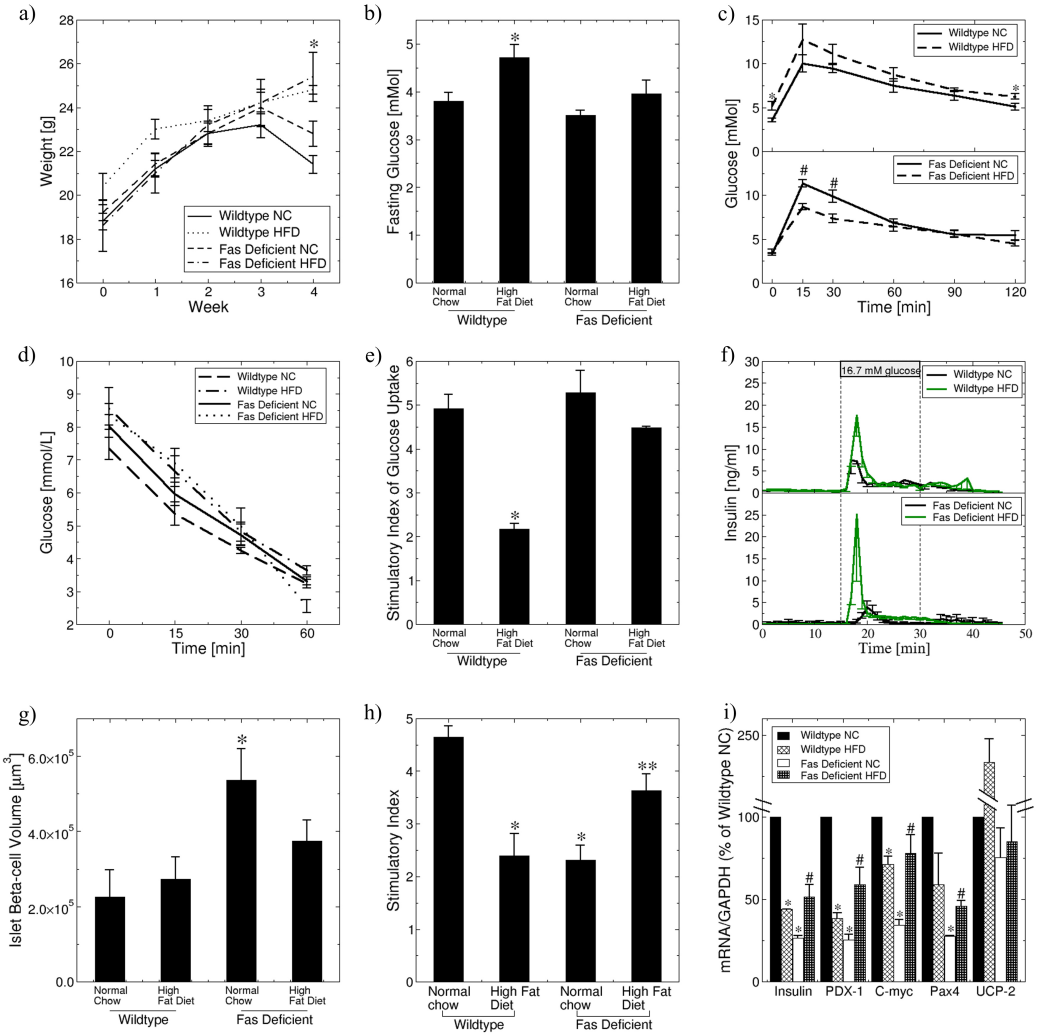
a)
Weight [g]
Week
Wildtype NC
Wildtype HFD
Fas Deficient NC
Fas Deficient HFD
b)
Fasting Glucose [mMol]
Normal Chow
High Fat Diet
Wildtype
Fas Deficient
c)
Glucose [mMol]
Time [min]
Wildtype NC
Wildtype HFD
Fas Deficient NC
Fas Deficient HFD
d)
Glucose [mmol/L]
Time [min]
e)
Stimulatory Index of Glucose Uptake
f)
Insulin [ng/ml]
16.7 mM glucose
Time [min]
g)
Islet Beta-cell Volume [µm3]
h)
Stimulatory Index
Normal chow
High Fat Diet
i)
mRNA/GAPDH (% of Wildtype NC)
Insulin
PDX-1
C-myc
Pax4
UCP-2

**Figure 5.1:** High fat diet improves insulin secretion in Fas deficient mice without development of insulin resistance. **(a)** Body weight of Fas deficient and wildtype mice placed on a high fat diet and on normal chow for 4 weeks and weighed weekly. **(b)** Fasting blood glucose levels after 12 hours of fasting. **(c)** Blood glucose levels following i.p. injection of glucose (2 mg/g body weight) in wildtype mice on normal chow (NC) vs. wildtype on a high fat diet (HFD), upper panel, and Fas deficient on normal chow (NC) vs. Fas deficient on a high fat diet (HFD), lower panel. * indicates $p < 0.05$, wildtype NC vs. wildtype HFD; # denotes $p < 0.05$, Fas deficient NC vs. Fas deficient HFD; $n = 10$ for each group. **(d)** Blood glucose levels following i.p. injection of insulin (0.75 mU/kg) in male Fas deficient versus wildtype mice on normal chow (NC) and high fat diet (HFD); $n = 10$ for each group. **(e)** Insulin-stimulated 2-Deoxyglucose uptake in adipocytes isolated from Fas deficient and wildtype mice on normal chow and high fat diet. Data are the means of 2 separate experiments, each in sextuplet.* indicates $p < 0.05$ vs. wildtype normal chow. **(f)** Glucose induced insulin secretion in perfused pancreata from Fas deficient and wildtype mice. Pancreata were perfused with basal solution (2.8 mM glucose) for 30 min before perfusate was collected (from time 0). Glucose was increased to 16.7 mM glucose for the indicated period, $n = 3$ for each group. **(g)** Pancreatic sections of Fas deficient and wildtype mice on a high fat diet or a normal chow diet were stained for insulin and glucagon. The area of the islets were measured using analySISTM computer software. **(h)** Stimulatory index of insulin secretion during successive 1 h incubation at 3.3 mM glucose (basal) and 16.7 mM glucose (stimulated) following a 4 day culture period of islets isolated from Fas deficient and wildtype mice on normal chow and high fat diet. Data are the means of 3 separate experiments, each in triplicate. * indicates $p < 0.05$ vs. wildtype normal chow, ** indicates $p < 0.05$ vs. Fas deficient normal chow. **(i)** Quantitative RT-PCR detection of insulin, PDX-1, c-myc, Pax4 and uncoupling protein 2 (UCP-2) mRNA expression. Total RNA was isolated from Fas deficient and wildtype islets of mice on normal chow and high fat diet. The level of mRNA expression was normalized to GAPDH and the results expressed as percentage of wildtype normal chow islet mRNA levels; $n = 3$ for each group. * denotes $p < 0.05$.

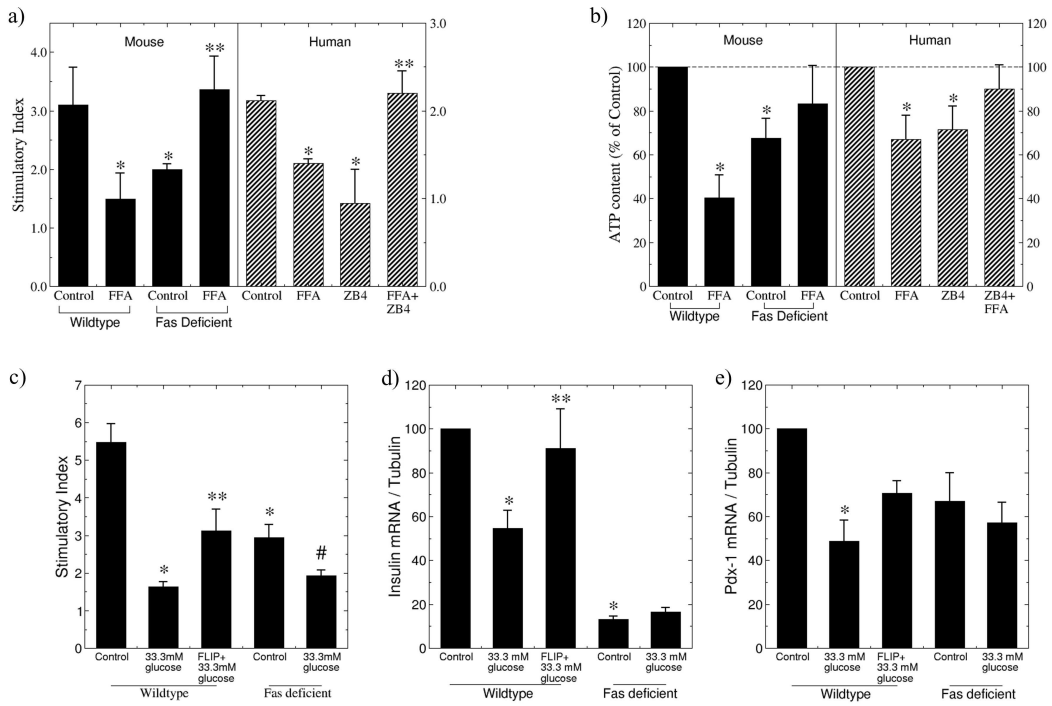


**Figure 5.2:** Fatty acids improve insulin secretion and chronic hyperglycaemia does not impair β-cell function in Fas deficient islets. **(a)** Stimulatory index of insulin secretion during successive 1 h incubation at 3.3 mM glucose (basal) and 16.7 mM glucose (stimulated) following a 2 day culture period of islets isolated from Fas deficient and wildtype mice (left panel) and human cadaveric donors (right panel)with palmitic/oleic acid. The human islets were exposed to 500 ng/ml isotype IgG (control) or 500 ng/ml antagonistic anti-Fas antibody (ZB4). **(b)** After a 4 day culture period, wildtype and Fas deficient mouse islets (left panel) and human islets cultured in the presence of 500 ng/ml isotype IgG (control) or 500 ng/ml antagonistic anti-Fas antibody (ZB4) (right panel), were incubated successively for 30 min in 2.8 mM glucose followed by an additional 10 min in 16.7 mM glucose and analysed for stimulated ATP-content. * indicates $p < 0.001$. Data are means of percentage relative to control for 3 separate experiments, each in triplicate. **(c)** Stimulatory index of insulin secretion during successive 1 h incubation at 3.3 mM glucose and 16.7 mM glucose following an 8 day culture period of islets isolated from wildtype and Fas deficient mice. Islets were chronically treated with 33.3 mM glucose only or transfected with a vector encoding for FLIP and then exposed to 33.3 mM glucose. **(d–e)** Quantitative RT-PCR detection of insulin and PDX-1 mRNA expression. The level of mRNA expression was normalized to tubulin and the results expressed as mRNA levels relative to controls. $n = 3$ to 4 independent experiments. * denotes $p < 0.05$ relative to wildtype controls and # $p < 0.05$ relative to wildtype 33.3 mM glucose.

### Fatty acids improve the function of Fas deficient β-cells which are protected from lipotoxicity

Free fatty acids (FFA) decreased the glucose-induced stimulatory index in wildtype islets, but improved the glucose-induced stimulatory index in Fas deficient islets (Fig. 5.2 a). This confirmed the in vivo results. Human islets treated with FFA showed an impairment in function, but treatment with the antagonistic anti-Fas antibody, ZB4, and FFA abrogated these effects (Fig. 5.2 a, right panel). As a possible mechanism, we looked at mitochondrial function, and in both human and mouse islets, there was a decrease in ATP content in wildtype/control islets treated with FFA, whereas there was a not significant increase in ATP content in the Fas deficient/ZB4 treated islets with FFA (Fig. 5.2 b).

### FLIP reverses glucotoxic effects on wildtype islets

There was a significant decrease in insulin secretion in wildtype islets exposed to 33.3 mM glucose whereas there was no significant decrease in insulin secretion in the Fas deficient islets (Fig. 5.2 c). The decrease in glucose stimulated insulin secretion in wildtype mice, could be improved when the islets were transfected with FLICE inhibitory protein (FLIP), which is found downstream of Fas. With gene expression analysis, we saw that the decrease in β-cell function in wildtype islets exposed to high glucose was due to a significant decrease in insulin and PDX-1 mRNA. FLIP increased both insulin and PDX-1 mRNA and improved the function. There were no glucose-induced changes in insulin or PDX-1 mRNA in the Fas deficient islets (Fig. 5.2 d and Fig. 5.2 e).

## 5.4 Discussion

Diet-induced obesity is a well-known mechanism for inducing diabetes in mice [11]. In our mouse model, we were able to induce increased fasting glucose, impaired glucose tolerance and insulin resistance after four weeks on a high fat diet, with no significant changes in β-cell mass. Fas deficient mice on the high fat diet, had an increase in weight similar to the wildtype mice on a high fat diet, but did not develop insulin resistance, or impaired fasting glucose and surprisingly had an improved glucose tolerance. The increase in glucose-induced insulin secretion in the Fas deficient mice after a high fat diet compared to the Fas deficient mice on a normal chow diet, could be due to the fact that no insulin resistance developed. Insulin resistance occurs when insulin is unable to induce the uptake of glucose by peripheral tissues such as muscle and fat. This results in increased circulating glucose, which in turn induces apoptosis and impairs β-cell function via IL-1βand Fas [2]. The glucotoxic effects which occurred in the wildtype mice on a high fat diet included decreased insulin and PDX-1 mRNA as well as decreased glucose-induced insulin secretion, therefore, did not occur in the Fas deficient mice on a high fat diet. On the contrary, the Fas deficient mice on the high fat diet had increased insulin and PDX-1 mRNA. Transfection of wildtype isolates with FLIP, which is found downstream of Fas, reversed the glucotoxic effects by increasing both insulin and PDX-1 mRNA. Since the

improvement in β-cell function in the Fas deficient mouse islets as well as in human islets treated with an antagonistic anti-Fas antibody, ZB4, was also evident in the in vitro studies, more factors appear to play a role. The production of mitochondrial ATP is critical for glucose-stimulated insulin secretion, therefore we investigated uncoupling protein 2 (UCP-2), the only uncoupling protein present in islets and a known regulator of mitochondrial ATP production [12, 13]. In the wildtype mice on a high fat diet, UCP-2 was significantly increased and ATP was significantly decreased after the high fat diet, whereas in Fas deficient mice on a high fat diet UCP-2 and ATP remained unchanged. The increased UCP-2 in the wildtype mice possibly increased proton leak and decreased the mitochondrial ATP production, which in turn decreased glucose-stimulated insulin secretion. When exposed to high glucose for a prolonged time period, Fas deficient islets proved to be impervious to glucotoxicity, since there were no changes in β-cell function or in the insulin and PDX-1 mRNA. It therefore appears that regulation of the Fas/FLIP pathway could be a tool used in the fight against type 2 diabetes.

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